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(54) Title: NOVEL HUMAN CYTOMEGALOVIRUS DNA CONSTRUCTS AND USES THEREFOR			
(57) Abstract Novel DNA molecules for <i>in vitro</i> and <i>in vivo</i> expression of HCMV gB, gB transmembrane-deleted derivatives, pp65, pp150, and IE-exon-4 proteins are described. Preferably, the molecules are plasmids. Also described are methods of using these DNA molecules to induce immune responses to HCMV, and the use of a plasmid of the invention to prime immune responses to HCMV vaccines.			

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NOVEL HUMAN CYTOMEGALOVIRUS DNA CONSTRUCTS
AND USES THEREFOR

Field of the Invention

This invention relates generally to compositions
5 useful in preventing and treating human cytomegalovirus
infection.

Background of the Invention

Cytomegalovirus (CMV) is one of a group of highly
host specific herpes viruses that produce unique large
10 cells bearing intranuclear inclusions. The envelope of
the human cytomegalovirus (HCMV) is characterized by a
major glycoprotein complex termed gB or gCI, which was
previously referred to as gA.

Infection with HCMV is common and usually
15 asymptomatic. However, the incidence and spectrum of
disease in newborns and immunocompromised hosts
establishes this virus as an important human pathogen.
HCMV has also been suggested to be an important co-factor
in the development of atherosclerosis and restenosis
20 after angioplastic surgery.

Several HCMV vaccines have been developed or are in
the process of development. Vaccines based on live
attenuated strains of HCMV have been described. [See,
e.g., S. A. Plotkin et al, Lancet, 1:528-30 (1984); S. A.
25 Plotkin et al, J. Infect. Dis., 134:470-75 (1976); S. A.
Plotkin et al, "Prevention of Cytomegalovirus Disease by
Towne Strain Live Attenuated Vaccine", in Birth Defects,
Original Article Series, 20(1):271-287 (1984); J. P.
Glazer et al, Ann. Intern. Med., 91:676-83 (1979); and U.
30 S. Patent 3,959,466.] A proposed HCMV vaccine using a
recombinant vaccinia virus expressing HCMV glycoprotein B
has also been described. [See, e.g., Cranage, M. P. et
al, EMBO J., 5:3057-3063 (1986).] However, vaccinia
vaccines are considered possible causes of encephalitis.
35 Other recombinant HCMV vaccines have been described.

See, e.g., G. S. Marshall et al, J. Infect. Dis.,
162:1177-1181 (1990); K. Berencsi et al, J. Gen. Virol.,
74:2507-2512 (1993), which describe adenovirus-HCMV
recombinants.

5 There remains a need in the art for additional
compositions useful in preventing CMV infection by
enhancing immune responses to HCMV vaccines and
generating neutralizing antibody and/or cellular
responses to CMV in the human immune system.

10 Summary of the Invention

The present invention provides a series of DNA
molecules expressing human cytomegalovirus (HCMV) genome
fragments, which are particularly useful in inducing
HCMV-specific immune responses.

15 Thus, in one aspect, the invention provides a DNA
molecule which is non-replicating in mammals and which
comprises at least one human cytomegalovirus antigen
which is operably linked to regulatory sequences which
express the antigen in the mammal. Advantageously, the
20 antigen elicits an immune response in said mammal. In
one preferred embodiment, the DNA molecule is a plasmid.

In another aspect, the invention provides a plasmid,
pTet-gB, containing the portion of the HCMV genome (UL55)
encoding gB. This plasmid further contains a
25 tetracycline regulatable HCMV-immediate early promoter,
which is useful in controlling expression of gB. Another
plasmid of the invention encoding the full-length gB
subunit protein is a pΔRC-gB plasmid.

Yet another plasmid of the invention, pΔRC-gB₆₈₀,
30 contains the portion of the HCMV genome encoding the N-
terminal 680 amino acids of the gB protein (gB₁₋₆₈₀).

The pΔRC-pp65 plasmid of the invention contains the
portion of the HCMV genome (UL83) encoding the HCMV pp65
tegument protein. The pΔRC-pp150 plasmid contains the

portion of the HCMV genome (UL32) encoding the HCMV pp150 tegument protein.

The pARC-exon-4 contains the portion of the HCMV genome (truncated UL123) encoding HCMV immediate-early (IE) exon-4.

In yet another aspect, the present invention provides an immunogenic composition of the invention comprising at least one of the DNA molecules of the invention and a carrier.

In still another aspect, the present invention provides a method of inducing HCMV-specific immune responses in an animal by administering to the animal an effective amount of an immunogenic composition of the invention. Preferably, this composition contains pARC-gB₆₈₀, pTet-gB and/or pARC-pp65.

In yet a further aspect, the present invention provides a method of priming immune responses to a selected human cytomegalovirus immunogenic composition by administering an immunogenic composition of the invention prior to administration of the second immunogenic or vaccine composition.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 illustrates the construction of the pTet-gB plasmid.

Fig. 2 is a graph illustrating the results of pp65-specific CTL responses in BALB/c mice immunized with pARC-pp65. The circle represents VacWR-pp65-infected MC57 (MHC-mismatched) target cells; the diamond represents WT-Vac-infected P-815 cells; and the square represents VacWR-pp65-infected P-815 (MHC-mismatched) target cells.

Fig. 3A-3E provides the full-length DNA and amino acid sequences [SEQ ID NO:1 and 2] of a human cytomegalovirus virus gB gene.

5 Fig. 4A - B provide the full-length DNA and amino acid sequences [SEQ ID NO:3 and 4] of a human cytomegalovirus immediate-early exon-4.

Fig. 5 provides the full-length DNA and amino acid sequences of a human cytomegalovirus phosphoprotein (pp) 65 gene Towne strain on the top line [SEQ ID NO: 5 and
10 6], and, on the bottom line, the sequence of the pp65-AD169 strain where it differs from the Towne strain [SEQ ID NO: 7 and 8].

Fig. 6A - B provide the full-length DNA and amino acid sequences [SEQ ID NO: 9 and 10] of a human
15 cytomegalovirus phosphoprotein (pp) 150 gene, AD169 strain.

Fig. 7A provides a circular map of the eukaryotic expression vector pCB11.

Fig. 7B provides a circular map of pCBgB.

20 Fig. 7C provides a circular map of pCBgB Δ tm.

Fig. 8 provides a schematic representation of the gB protein (top line) and of its homolog which is deleted of the transmembrane domain (bottom line).

Fig. 9 is a graph illustrating the anti-gB titers in
25 sera of BALB/c mice immunized with plasmids pCBgB and pCBgB Δ tm intramuscularly (IM) and intradermally (ID).

Detailed Description of the Invention

The present invention provides DNA molecules useful for in vitro and in vivo expression of antigenic
30 fragments of the HCMV genome. Particularly desirable antigens include full-length and transmembrane-deleted fragments of gB such as gB₁₋₆₈₀, pp65, pp150, and IE-exon-4. Preferably, the DNA molecules of the invention are plasmids. The inventors have found that these DNA

molecules induce HCMV-specific immune responses, including ELISA and neutralizing antibodies and cytotoxic T lymphocytes (CTL), and are further useful in priming immune responses to subsequently administered HCMV immunogens and vaccines.

Thus, in one embodiment, the present invention provides a DNA molecule containing at least one HCMV antigen under the control of regulatory sequences which express the antigen in vivo or in vitro. Desirably, the DNA molecule is incapable of replicating in mammals. In a particularly desirable aspect of this embodiment, the DNA molecule is a plasmid.

As defined herein, an HCMV antigen includes a portion of the HCMV genome or a protein or peptide encoded thereby which induces an immune response in a mammal. Desirably, the immune response induced is HCMV-specific and protective. However, non-protective immune responses are also useful according to the invention, e.g., for priming immune responses. Currently, preferred HCMV antigens include full-length gB, a fragment or derivative of gB which lacks at least the transmembrane domain, pp65, pp150, and the immediate-early exon-4. Other suitable antigens may be readily selected by one of skill in the art.

The exemplary DNA molecules of invention, described herein, have been constructed using gene fragments derived from the Towne strain of HCMV. The Towne strain of HCMV, is particularly desirable because it is attenuated and has a broad antigenic spectrum. This strain is described in J. Virol., 11 (6): 991 (1973) and is available from the ATCC under accession number VR-977. The Ad169 strain is also available from the ATCC, under accession number VR-538. However, other strains of CMV useful in the practice of this invention may be obtained

from depositories like the ATCC or from other institutes or universities, or from commercial sources.

Thus, the CMV gene fragment encoding the desired protein (e.g., gB, pp65, pp150) or protein fragment (e.g., gB₁₋₆₈₀ or IE-exon-4) may be isolated from known HCMV strains. See, e.g., Mach et al, J. Gen. Virol., 67:1461-1467 (1986); Cranage, M. P. et al, EMBO J., 5:3057-3063 (1986); and Spaete et al, Virol., 167:207-225 (1987), which provide isolation techniques. For example, using a known HCMV sequence, the desired HCMV gene or gene fragment [e.g., pp65 (UL83)] is PCR amplified, isolated, and inserted into the plasmid vector or other DNA molecule of the invention using known techniques. Alternatively, the desired CMV sequences can be chemically synthesized by conventional methods known to one of skill in the art, purchased from commercial sources, or derived from CMV strains isolated using known techniques.

If desired, the DNA molecules of the invention may contain multiple copies of the HCMV gene or gene fragment. Alternatively, the recombinant plasmid may contain more than one HCMV gene/gene fragment, so that the plasmid may express two or more HCMV proteins. For example, as shown herein, the presence of both gB- and pp65-specific ELISA antibodies and pp65-specific CTL in the mice inoculated with pTet-gB and pΔRC-pp65 in a mixture indicates that gB and pp65 do not mutually block antigen presentation or B and T cell stimulation when expressed in the same cells or in close proximity. Thus, gB (or gB₆₈₀) and pp65 proteins are particularly well suited for incorporation into a plasmid which expressed both protein (termed herein a chimeric vector). Thus, one particularly desirable embodiment of the present invention provides a DNA molecule containing the gB and the pp65 antigens. In another particularly desirable

embodiment, the DNA molecule contains a transmembrane-deleted gB fragment or derivative (e.g, gB₆₈₀ or gBΔtm) and the pp65 antigens.

5 In the construction of the DNA molecules of the invention, one of skill in the art can readily select appropriate regulatory sequences, enhancers, suitable promoters, secretory signal sequences and the like. In the examples below, the plasmids have been provided with a tetracycline repressor from *E. coli*. However, if
10 desired, the plasmid or other DNA molecule may be engineered to contain another regulatable promoter, which "turns on" expression upon administration of an appropriate agent (e.g., tetracycline), permitting regulation of *in vivo* expression of the HCMV gene
15 product. Such agents are well known to those of skill in the art. The techniques employed to insert the HCMV gene into the DNA molecule and make other alterations, e.g., to insert linker sequences and the like, are known to one of skill in the art. See, e.g., Sambrook et al,
20 "Molecular Cloning. A Laboratory Manual" (2d edition), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

In one embodiment, the DNA molecules of the invention are plasmids. One exemplary plasmid is pTet-gB.
25 gB. Construction of this plasmid is described in more detail below. Plasmid TetotTA-gB contains the gene from HCMV (the unique long (UL) 55) encoding the full-length gB subunit protein and a tetracycline regulatable HCMV-immediate early promoter which controls expression of gB.
30 For convenience, the sequences of the HCMV gene fragment encoding the full-length gB protein which were used in the examples below are provided in Fig. 3A-3E [SEQ ID NO: 1 and 2]. As discussed herein, this invention is not limited to this strain of HCMV. pTet-gB has been found
35 to be useful alone, and in conjunction with the other DNA

molecules of the invention, and particularly the pARC-pp65 plasmid described below. pTet-gB is also particularly useful for priming immune responses to subsequently administered HCMV immunogenic compositions and vaccines.

The pTetotTA-gB plasmid has been deposited pursuant to the Budapest Treaty, in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. This deposit, designated ATCC 98029, was made on April 23, 1996 and is termed herein, pTet-gB.

Other plasmids provided herein, pARC-gB and pCBgB, also contain the HCMV gene encoding the gB protein. As demonstrated below, these DNA plasmids have been found to be highly potent immunogens for HCMV. See Examples 8 and 14.

Another plasmid of the invention, pARC-gB₆₈₀ contains the portion of the HCMV gene encoding the N-terminal 680 amino acids of the gB protein and is capable of expressing this fragment *in vivo* or *in vitro*. This gB fragment is designated herein gB₁₋₆₈₀. As illustrated in Figure 3A-E [SEQ ID NO:2], the full-length gB subunit protein consists of 907 amino acids. This plasmid, which expresses a secreted form of gB, has been found to be a more potent immunogen than the plasmids expressing the full-length gB.

Also provided herein is plasmid pCDgB Δ tm, which contains a deletion of the gB transmembrane region. This plasmid has been found to induce HCMV-specific neutralizing antibodies (see Example 14) and to be a more potent immunogen than the corresponding DNA plasmid encoding full-length gB.

Plasmid pARC-exon-4 contains the portion of the HCMV immediate-early (IE) gene encoding HCMV IE-exon-4 and is capable of expressing the gene product. The HCMV IE-exon-4 gene fragment has been described in

international patent application PCT/US94/02107,
published August 18, 1994, which is incorporated by
reference herein. The IE gene and the intron/exon
junctions for Towne strain HCMV are provided in Stenberg
5 et al, J. Virol., 49:190-199 (1984), and are available
from GenBank under accession number K01484, M11828-30.
The sequences of the IE-exon-4 gene fragment, Towne
strain, are provided in Fig. 4A - B [SEQ ID NO: 3 and 4],
for convenience. This invention is not limited to the
10 use of the IE-exon-4 sequences from this viral strain.

Plasmid pARC-pp65 contains the HCMV gene encoding
the HCMV phosphoprotein (pp) 65 tegument protein and is
capable of expressing pp65 *in vivo* or *in vitro*. As
described herein, immunization with pARC-pp65 induced a
15 reduction of virus titers in the mouse lung after
intranasal challenge with vaccinia recombinants carrying
the pp65 gene, suggesting the protective function of
cell-mediated immunity in lung after DNA immunization.
Further, in contrast to a prior art pp65-containing
20 plasmid construct which induced ELISA antibodies in only
about 60% of inoculation mice, nearly 100% of mice
inoculated with pARC-pp65 responded with pp65-specific
ELISA antibodies. The sequences of the pp65 gene, Towne
and AD169 strains, have been described in H. Pande et al,
25 Virol., 181(1):220-228 (1991) and are provided in Fig. 5
[SEQ ID NO: 5 - 8] for convenience. pp65 sequences may
be readily isolated using known techniques from other
HCMV strains, or obtained from commercial sources. The
strain from which the pp65 sequences are derived is not a
30 limitation on the present invention.

Plasmid pARC-pp150 contains the portion of the HCMV
gene encoding the HCMV pp150 tegument protein and is
capable of expressing pp150 *in vivo* or *in vitro*. The
sequences of the pp150 gene, Ad169 strain, have been
35 described in G. Jahn et al, J. Virol., 61(5):1358-1367

(1987) and are provided in Fig. 6A - B for convenience [SEQ ID NO: 9 and 10]. pp150 sequences may be readily isolated using known techniques from another HCMV strain, or obtained from commercial sources. The strain from
5 which the pp150 sequences are derived is not a limitation on the present invention.

The DNA molecules, and particularly the plasmids described herein, may be used for expression of the gB, gB₁₋₆₈₀ fragment, pp65, pp150, or IE-exon-4 *in vitro*. The
10 molecules are introduced by conventional means into the desired host cell [see, Sambrook et al, cited above]. Suitable host cells include, without limitation, bacterial cells, mammalian cells and cell lines, e.g., A549 (human lung carcinoma) or 293 (transformed human
15 embryonic kidney) cells.

The host cell, once transfected with the recombinant plasmid (or other DNA molecule) of the present invention, is then cultured in a suitable medium, such as Minimal Essential Medium (MEM) for mammalian cells. The culture
20 conditions are conventional for the host cell and allow the expressed HCMV protein, e.g., gB, to be produced either intracellularly, or secreted extracellularly into the medium. Conventional protein isolation techniques are employed to isolate the expressed subunit from the
25 selected host cell or medium.

Alternatively, transfected host cells are themselves used as antigens, e.g., in *in vitro* immunological assays, such as enzyme-linked immunosorbent assays (ELISA). Such assay techniques are well known to those of skill in the
30 art.

In yet another embodiment, one or more of the DNA molecules (e.g., plasmids) described herein may be used directly as immunogens in an immunogenic composition or directly for priming the immune response to a
35 subsequently administered immunogenic or vaccine

composition. According to this embodiment of the invention, the DNA molecule (e.g., plasmid) containing the HCMV gene or gene fragment is introduced directly (i.e., as "naked DNA") into the animal by injection. The
5 DNA molecule of the invention, when introduced into an animal, transfects the host's cells and produces the CMV protein in those cells. Methods of administering so-called 'naked DNA', are known to those of skill in the art. [See. e.g., J. Cohen, Science, 259:1691-1692 (March
10 19, 1993); E. Fynan et al, Proc. Natl. Acad. Sci., 90:11478-11482 (Dec. 1993); J. A. Wolff et al, Biotechniques, 11:474-485 (1991); International Patent Application PCT WO94/01139, which are incorporated by
15 reference herein for purposes of described various 'naked DNA' delivery methods.]

The preparation of a pharmaceutically acceptable immunogenic composition, having appropriate pH, isotonicity, stability and other conventional characteristics is within the skill of the art.
20 Currently, in a preferred embodiment, one or more of the recombinant plasmids (or other DNA molecules) of the invention is suspended in an acceptable carrier such as isotonic water, phosphate buffered saline, or the like. Optionally, although currently less preferred, such a
25 composition may contain other components, such as adjuvants, e.g., aqueous suspensions magnesium hydroxides.

An effective amount of an immunogenic composition of the invention preferably contains between 10 μ g and 10
30 mg, and preferably between about 80 μ g and 150 μ g of DNA of the invention per inoculation. Desirably, for each inoculation, the DNA of the invention is formulated in about 100 μ l of a suitable carrier. In a particularly preferred embodiment, each patient is administered 100 μ g
35 DNA, which is administered three times at about 4 week

intervals. Alternatively, the dosage regimen involved in the method for immunizing with the recombinant DNA molecule (e.g., plasmid) of the present invention can be determined considering various clinical and environmental factors known to affect vaccine administration. For example, following a first administration of an immunogenic composition of the invention, boosters may be administered approximately 2- to 15-weeks later. These boosters may involve an administration of the same immunogenic composition as was first administered, or may involve administration of an effective amount of another immunogenic composition of the invention. Additional doses of the vaccines of this invention may also be administered where considered desirable by the physician.

In another aspect, the present invention provides a method of inducing HCMV-specific immune responses in an animal. The method involves administering to an animal an effective amount of an immunogenic composition containing one or more of the DNA molecules of the invention, as described above. The immunogenic composition is administered by any suitable route, including oral, nasal routes, subcutaneous and intraperitoneal. However, currently preferred are the intramuscular and intradermal routes of administration.

In a particularly preferred embodiment of this aspect, the method of inducing an HCMV-specific immune response of the invention involves the administration of one or more immunogenic compositions of the invention. These compositions may be formulated so as to contain a single DNA molecule of the invention, or may contain mixtures of the DNA molecules of the invention. In one desirable embodiment, the composition contains pΔRc-gB₆₈₀ or pCBgBΔtm. In another desirable embodiment, the composition contains a plasmid containing pp65 according to the invention. As illustrated in the examples below,

administration of pΔRC-pp65 has been found to induce a potent HCMV-specific immune response. In another desirable embodiment of the invention, the combined administration of pTet-gB and pΔRC-pp65 invention (which
5 may be formulated in a single composition, or preferably, administered separately) induces potent HCMV-specific ELISA and neutralizing antibodies to both proteins. In yet another desirable embodiment, the present invention provides a composition containing a chimeric plasmid
10 which expresses pp65 and gB₆₈₀ or gB. Yet another desired embodiment involves combined administration of pΔRC-gB₆₈₀ and pΔRC-pp65.

In another aspect of this invention, a method of priming immune responses to a human cytomegalovirus
15 immunogenic or vaccinal composition is provided. This method involves administering an immunogenic composition of the invention prior to administration of a second immunogenic or vaccinal composition. Desirably, an effective amount of an immunogenic composition of the
20 invention, e.g., containing pTet-gB, is administered between about 4 and 15 weeks prior to administration of the immunogenic or vaccinal composition. The second immunogenic or vaccinal composition, for which the immune response is enhanced or primed by the method of the
25 invention, may be an immunogenic composition of the invention or a conventional immunogenic or vaccine composition. For example, such a composition may contain one or more HCMV proteins (e.g., the isolated, purified gB protein described in the examples below), a whole
30 virus (e.g., semipurified Towne strain HCMV virion), or recombinant HCMV viruses. Suitable recombinant viruses are well known to those of skill in the art and include, e.g., the Ad-gB virus [G. Marshall et al, (1990), cited above, and EP 389 286; the Ad-gB-IE-exon-4 virus [WO
35 94/17810]; the Ad-gB fragment viruses [WO 94/23744].

Other suitable HCMV vaccinal compositions are well known to those of skill in the art.

These examples illustrate the preferred methods for preparing and using the plasmids of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Construction of pTet-gB plasmid

The full-length HCMV-gB gene was obtained from the plasmid pAd-gB [Marshall et al., J. Infect. Dis., 162:1177-1181 (1990)] by XbaI-XbaI-digestion.

The full length HCMV-gB was inserted into the plasmid pUHD10-3 [Gossen and Bujard, Proc. Natl. Acad. Sci. USA, 12:5547-5551 (1992)]. This plasmid contains:

- (a) a tetracycline regulatable promoter (HCMV minimal promoter, - 53 relative to the start site, with heptamerized tet-operon derived from the regulatory region of tet^R - gene of transposon -10);
- (b) a multiple cloning site (including an XbaI site); and
- (c) an SV40 polyadenylation signal downstream of the polycloning site.

After inserting the HCMV-gB (referred to as pTet0-gB), the plasmid was digested with Hind^{III} followed by blunt-ending, then digested with PvuI and the fragment containing the tetracycline regulatable promoter-HCMV-gB-SV40 polyA signal sequences was isolated and inserted into the plasmid pUHD15-1 [Gossen and Bujard, cited above]. This latter plasmid (hereafter referred to as pTtA) contains the HCMV-IE promoter-enhancer which constitutively drives the tTAgene followed by the SV40 polyA signal. The tTA-gene codes for a fusion protein consisting of the tetracycline repressor from *E. coli* and the carboxy-terminal 130 amino acids of the herpes simplex virus protein 16 gene (HSV

VP-16). This fusion protein is a powerful transactivator of the tetracycline regulatable promoter of pTeto (which drives the HCMV-gB gene), because of the specific and high affinity attachment of the tetracycline repressor to the tetracycline operator sequences ensures the activation of transcription from the minimal HCMV promoter by the transactivator domain of HSV VP-16 gene (fused to the tetracycline repressor). The gene activation is specific for the pteto promoter. In the presence of low, non-toxic concentration of tetracycline (1 μ g/ml or less), however, the transactivation is switched off, since tetracycline prevents the attachment of the tetracycline repressor to the teto sequences and no or very low gene expression is allowed (i.e., only the minimal HCMV promoter basal activity which is negligible in almost all cell types investigated so far).

To obtain the gB-expression plasmid regulatable by tetracycline, ptTA was cut just upstream of the HCMV-IE promoter/enhancer by *Xho*I, blunt-ended and cut with *Pvu*I. The large fragment containing the HCMV-IE promoter-enhancer-tTA fusion protein gene followed by the SV40 polyA signal and the *E. coli* sequences of the plasmid (i.e., the replication origin and the beta-lactamase genes) were isolated. This isolated fragment was ligated with the fragment of pUHD10-3 containing the gB gene by the competent blunt-end and *Pvu*I ends, resulting in the plasmid pteto-gB-tTA. The resulting plasmid contains both the transactivator and the HCMV-gB gene. The structure of the plasmid is, in addition to the *E. coli*-part, tetracycline-regulatable promoter (7 teto + minimal HCMV promoter) followed by the HCMV-gB gene, followed by the SV40 polyA signal, followed by the HCMV-IE promoter-enhancer, followed by the tTA gene and ending with the SV40 polyA signal.

The tetracycline-controllable expression system has been found to work correctly *in vivo* in the mouse as well [J. Dhawan et al, Somatic Cell and Molecular Genetics, 21:233-240 (1995)]. The pTet-gB plasmid is suitable to control naked DNA immunization. It is possible to give tetracycline to mice in their drinking water in concentrations not toxic for the animals but reaching sufficient levels able to regulate expression in muscle tissues [J. Dhawan et al., Somatic Cell and Molecular Genetics, 21: 233-240 (1995)]. By tetracycline treatment of transfected cultures or inoculated mice the time of antigen exposure can be manipulated. The silent presence of the inoculated plasmid can be tested. Without tetracycline treatment, however, this plasmid simply serves as a plasmid DNA immunogen or vaccine.

Example 2 - Construction of further Plasmids

A. Construction of pRC-gB

pRC/CMV (Invitrogen Corporation) contains the HCMV-IE promoter. The full length gB gene (XbaI-XbaI fragment from pAd5-gB) was obtained using conventional techniques [SEQ ID NO:1] and inserted into pRC/CMV according to manufacturer's directions. The resulting plasmid is termed herein pRC-gB.

B. Construction of pΔRC-gB

pΔRC/CMV was derived from pRC/CMV plasmid by deleting the PvuII 1290 - PvuII 3557 fragment to obtain more unique restriction sites. The full gB [SEQ ID NO:1], derived from the plasmid pAd-gB [Marshall et al., J. Infect. Dis., 162:1177-1181 (1990)], was subcloned using conventional techniques, inserted into pUC-8 (commercially available), then obtained as a HindIII-BamHI fragment and inserted into the HindIII-BamHI digested pΔRC/CMV vector. The resulting plasmid is termed pΔRC-gB.

C. Construction of pΔRC-gB₆₈₀

pΔRC-gB₆₈₀ expresses the N-terminal 680 amino acids of the gB protein [SEQ ID NO:2]. The plasmid was derived from pΔRC-gB, by deleting the C-terminal 227 amino acids of the gB by Xho-digestion, Klenow polymerase filling, removing the C-terminal portion of the gB gene, and religation of the 5400 bp fragment. The insert is approximately 2200bp.

Example 3 - Construction of pΔRC-pp65 and pΔRC-pp150

10 A. pΔRC-pp65

The plasmid pΔRC-pp65, which expresses the pp65 tegument protein of HCMV, was constructed as follows. H. Pande et al, Virology, 182(1):220-228 (1991), which provides the nucleotide sequences of the pp65 gene, is incorporated by reference herein [SEQ ID NO: 5 and 6].

15 The pp65 gene was isolated from the HCMV genome using conventional polymerase chain reaction techniques and inserted into a suitable expression plasmid. In this experiment, the 1696-bp pp65 gene was excised from the pUC-8-pp65 expression plasmid [Virogenetics] by NruI - BamHI digestion. The vector was blunt-ended with Klenow polymerase, digested with BamHI, and the pp65 gene inserted.

25 B. pΔRC-pp150

The plasmid, pΔRC-pp150, which expresses the pp150 tegument protein of HCMV, was constructed as follows. The pp150 gene was isolated from the HCMV genome using conventional polymerase chain reaction techniques and inserted into a suitable expression plasmid. One of skill in the art can readily isolate this gene from a desired HCMV strain making use of the published sequences in G. Jahn et al, J. Virol., 61(5):1358-1367 (1987) (which provides the nucleotide

sequences of the Ad169 HCMV pp150 gene and is incorporated by reference herein). See, also Fig. 6A-B herein [SEQ ID NO: 9 and 10].

5 In this experiment, the isolated HCMV-pp150 gene was inserted into the XbaI-restricted pΔRCd [Virogenetics]. The insert is approximately 3200 bp [SEQ ID NO: 10].

Example 4 - Construction of pΔRC-IE-Exon-4

10 The plasmid, pΔRC-IE-Exon-4, which expresses the HCMV-IE exon4 product [SEQ ID NO:4], was constructed as follow. The gene was obtained from pAd5-IE-Exon-4 [International Patent Application WO94/17810, published August 18, 1994 and Berencsi et al., Vaccine, 14:369-374 (1996)], by XbaI-digestion [SEQ ID NO:3]. The insert is
15 1230 bp.

Example 5 - Production of plasmid preparation stocks

E. coli DH5α competent cells (Gibco BRL, Gaithersburg, MD) were transformed with the constructed plasmids. Purified plasmid preparations were prepared on
20 Plasmid Giga Kits (Qiagen Inc. Chatsworth, CA).

Example 6 - Expression of HCMV-proteins after transient transfection of 293 cells with the purified plasmid preparations

25 Transient transfections were performed by the purified plasmid preparations, 1.5 μg/3x10⁵ cells, using lipofectamine (Gaithersburg, MD). Cells were tested for HCMV-protein expression 2 days after transfection by an immunofluorescence test as described in E. Gonczol et al, Science, 224:159-161 (1984). The antibodies used in this
30 test include the monoclonal pp65-specific Ab [VIROSTAT, Portland, Maine, stock # 0831], monoclonal gB-specific Ab [Advanced Biotechnologies, Columbia, MD], and anti-pp150

monoclonal Ab [Virogenetics Corporation]. The IE-Exon-4-specific monoclonal Ab P63-27 was provided by W. Britt, University of Alabama at Birmingham.

5 The pTet-gB plasmid expresses the full-length HCMV-gB gene under the control of a tetracycline regulatable HCMV-IE promoter. The other plasmids express the inserted gene in transfected 293 cells under the control of the HCMV-IE promoter. Expression of gB, pp65 and pp150 was found to be strong using all plasmids.

10 After transfection with pTet-gB, 10-12% and <1% of cells expressed gB protein in the absence and presence, respectively, of 1 μ g tetracycline [Tetracycline hydrochloride, Sigma, St. Louis, MO]. Sixty to seventy percent and 40-50% of cells transfected with p Δ RC-gB and p Δ gB₆₈₀ plasmids, respectively, expressed gB. pp65
15 protein was expressed in 70-80% of cells transfected with p Δ RC-pp65.

Example 7 - Immunization Procedures and Assay Methods

A. Immunization procedure

20 BALB/c or CBA mice were first pretreated i.m. with 100 μ l of Bupivacaine HCl [0.25% Sensorcaine-MPF (ASTRA Pharmaceutical Products, Inc. Westborough, MA)]. In some experiments, identified below, no Bupivacaine pretreatment was used. One day later DNA was inoculated
25 i.m. on the site of Bupivacaine infiltration. The dose for mice was 50-80 μ g plasmid DNA/ inoculation. Booster inoculations were given i.m. 2x, without pretreatment with Bupivacaine. Mice immunized with p Δ RC-gB plasmid were boosted 1 x. Mice were bled by retroorbital puncture
30 at the indicated times.

B. ELISA

Semipurified HCMV virions and purified gB proteins may be prepared by immunoaffinity column chromatography as described in E. Gonczol et al, J.

Virol., 58:661-664 (1986). Alternatively, one of skill in the art can readily obtain suitable virions and gB proteins by alternative techniques.

Semipurified HCMV virions (Towne strain) or
5 purified gB protein preparation were used as coating antigen for detection of gB-specific antibodies. OD values higher than mean OD values \pm 2SD of preimmune sera were considered positive, or OD values >0.05 , whichever was higher. Lysates of 293 cells transiently transfected
10 with pARC-pp65 were used as coating antigen for detection of pp65-specific antibodies, lysates prepared from untransfected 293 cells served as control antigen. OD values obtained on control antigen-coated wells were subtracted from OD values obtained on pp65 antigen-coated
15 wells and were considered positive if the resulting value was higher than 0.05.

C. Microneutralization assay

This assay was performed as described in E. Gonczol et al., J. Virol. Methods, 14:37-41 (1986). A
20 neutralizing titer higher than 1:8 was considered positive.

D. Cytotoxic T lymphocyte assay

This assay was performed as described in K. Berencsi et al., J. Gen Virol., 74:2507-2512 (1993).
25 Briefly, spleen cells of immunized mice were restimulated *in vitro* with VacWR-pp65-infected (m.o.i. = 0.2-0.5) autologous spleen cells (effector:stimulator ratio, 2.:1) for 5 days in 24-well plates. Cytolytic activity of nonadherent spleen cells was tested in a 4-h ^{51}Cr -release
30 assay. Target cells (P815 MHC class I-matched, MC57 MHC class I-mismatched) were infected with VacWR-pp65 or VT-Vac WR (m.o.i. = 4-8). Percentage of specific ^{51}Cr -release was calculated as [(cpm experimental release -

cpm spontaneous release)/(cpm maximal release - cpm spontaneous release) x 100]. A pp65-specific cytotoxicity higher than 10% was considered positive.

Example 8 - Induction of HCMV-Specific Immune Responses by the Plasmid Constructs Expressing the gB Protein

BALB/c mice were inoculated 2 times at 0 and 5 weeks with 80 µg pARC-gB preparation. Serum samples at 5, 9 and 19 weeks after the first inoculation were tested for HCMV-specific ELISA antibodies and neutralizing antibodies (NA). The results are provided in Table 1 below, in which the ELISA antigen used was semipurified virions. The OD of responders is provided as the mean±SD at a serum dilution of 1:80. Mean ± 2SD of the 6 preimmunization sera at a dilution of 1:80 gave an OD value of 0.080. "GM" indicates the geometric mean.

Table 1

pARC-gB induces HCMV-specific ELISA and neutralizing antibodies (antigen: semipurified virion).

20	weeks after first inoculation	No. of ELISA responders/ total	OD of resp. dil 1:80	No. of NA resp.	GM of NA
	0	0/6	0.036±0.022	0/6	NA
25	5	5/6	0.314±0.188	2/2	19
	9	6/6	1.387±0.810	6/6	34
	19	ND	ND	4/4	22

These data demonstrate that all mice responded with both ELISA antibody and NA after the booster inoculation. The pARC-gB plasmid seems to be a highly potent immunizing construct.

Table 2

pTet-gB and pARC-pp65 induces insert-specific ELISA antibodies

5	Mice Immunized With:	Weeks after first	# ELISA responders /total	OD* responders
		Inoc.		
10	pTet-gB	4	1/10	0.062
		8	9/10	0.277 ± 0.257
		13	7/7	0.530 ± 0.625
		21	6/6	0.503 ± 0.682
		31	5/6	0.451 ± 0.505
15	pARC-pp65	4	5/10	0.168 ± 0.070
		8	10/10	0.568 ± 0.387
		13	4/4	1.076 ± 0.216

* Mean OD ± SD of serum samples at dilution 1:40.

HCMV-specific ELISA antibodies were detected in 9 of 10 mice at 8 weeks after the first inoculation with pTet-gB (Table 2). HCMV neutralizing antibodies were detected in 4 of 10 mice, with titers between 1:16 and 1:48 (not shown). All mice immunized with the pARC-pp65 responded with pp65-specific ELISA antibodies. At 13 weeks (pp65- and gB-specific) and up to 31 weeks (gB-specific), OD values remained positive. In a separate experiment pp65-specific ELISA antibodies were also detected during the whole observation period (31 weeks) in 10 of the 10 immunized mice.

Example 9 - Induction of HCMV-Specific Immune Responses by the Plasmid Constructs Expressing pp65

To test whether the combination of the pTet-gB and pARC-pp65 results in reduced responses to the individual components, mice were immunized with both plasmids mixed together or inoculated separately. Groups of mice were inoculated with Bupivacaine (100 µl/mouse, 50 µl/leg),

and 2 days later, with either a mixture of both plasmids (80 µg of each DNA/mouse, 40 µg of each DNA/leg, 160 µg DNA/mouse) or each plasmid inoculated into two different legs (80 µg DNA of each plasmid/mouse, a total of 160 µg DNA/mouse inoculated in left and right legs). A similar booster was given 4 weeks later. The time course of both the gB- and pp65-specific ELISA antibody response was very similar in both groups, with nearly all mice developing antibodies by 8 or 13 weeks after the first inoculation (Table 3). In another experiment using the combination of the two plasmids, comparable OD values were observed up to 31 weeks after the first inoculation.

Table 3

pTet-gB and pΔRC-pp65 inoculated into the same animal induce gB and pp65-specific antibodies

Antigen, <u>Inoculation</u>	Weeks # gB- after ELISA 1st resp. <u>Inoc. /Total</u>	OD* of <u>responders</u>	# pp65- ELISA resp. <u>/Total</u>	OD of <u>Responders</u>
20 pTet-gB+ pΔRC-pp65, mixed	4 4/10	0.087±0.024	5/10	0.078±0.033
	8 10/10	0.220±0.143	10/10	0.400±0.321
	13 10/10	0.392±0.152	9/10	0.303±0.224
25 pTet-gB+ pΔRC-pp65, separately	4 8/10	0.076±0.021	6/10	0.210±0.124
	8 9/10	0.202±0.268	8/10	0.452±0.333
	13 10/10	0.309±0.202	8/10	0.308±0.212

* The mean OD ± SD of serum samples at dilution 1:40.

Of six mice inoculated with pΔRC-pp65 alone at a single site, 3 mice responded with pp65-specific lysis of target cells (Fig. 2). In a second similar experiment, 3 of 9 mice immunized with pΔRC-pp65 alone showed strong

pp65-specific CTL responses. pp65-specific CTL were also detected in 4 of 5 tested mice inoculated with the mixture of p Δ RC-pp65 and pTet-gB. When the p Δ RC-pp65 and pTet-gB were inoculated separately into two different
5 legs, 4 of 6 mice tested developed pp65-specific CTL response. These results establish that: 1) pp65-specific CTL responses are induced after DNA immunization; 2) there is no antigenic competition
10 between the gB and pp65 proteins in the induction of antibody and CTL responses; and 3) gB protein expression in the cells at the inoculation site does not interfere with the presentation of pp65-specific T cell epitopes by MHC class I molecules to T cells.

Example 10 - Priming effect of pTet-gB

15 One inoculation of naked plasmid DNA in mice did not result significant antibody responses in a high percentage of mice. To find out whether the immune system of the nonresponder mice was specifically primed by the DNA inoculation, mice inoculated with pTet-gB were
20 boosted 4 weeks later with either purified gB protein (5 μ g gB/mouse in Alum s.c.) or with the Towne strain of HCMV (20 μ g/mouse in Alum s.c.).

Table 4

Inoculation of mice with pTet-gB primes the immune system

	Antigen	wks after priming	No. of NA responder/all	GM of NA/ responder
5	Teto-gB/*	4	0/10	5
	Teto-gB	8	4/10	21
10	Teto-gB/*	4	0/10	4
	gB+Alu	8	8/10	77
	- / *	4	0/10	NA
	gB+Alu	8	1/10	16
	Teto-gB/**	12	1/5	16
	Towne+Alu	14	5/5	97
15	- / **	12	0/5	NA
	Towne+Alu	14	3/5	25

* second inoculations were given 4 weeks after the first inoculation

** Towne was given 12 weeks after the first inoculation

This data demonstrates that pTet-gB inoculation primes immune-responses. In other words, the combination of Teto-gB priming and gB+Alu or Towne+Alu booster gave higher number of responder mice and slightly higher NA titers than TetotTA-gB given 2 times.

Example 11 - DNA immunization decreases replication of the corresponding vaccinia recombinant in mice

Vaccinia virus recombinants expressing either HCMV-gB or pp65 were prepared using the methods described in WO 94/17810, published August 18, 1994. Briefly, the VacWR-gB and VacWR-pp65 recombinants were constructed as described [Gonczol et al, Vaccine, 9:631-637 (1991)], using the L variant of the neurovirulent WR strain of vaccinia virus as vector [Panicali et al, J. Virol.,

37(3):1000-1010 (1981)] and the gB or pp65 genes (HCMV Towne strain) as inserts cloned into the nonessential BamHI site in the HindIII F region [Panicali and Paoletti, Proc. Natl. Acad. Sci., 79:4927-4931 (1982)]
5 under the control of the vaccinia H6 early/late promoter. Vaccinia recombinant viruses and the parental wild-type WR strain were grown on Vero cells and purified as described [Gonczol et al, cited above].

After plasmid immunization, vaccinia virus
10 recombinants expressing either HCMV-gB or pp65 were used for challenge in the model described in WO 94/23744, published October 27, 1994. Vaccinia virus WR strain replicates in mouse lung after intranasal inoculation and immune protection can be evaluated by virus titrations of
15 the lung. Eight-week old female CBA and BALB/c mice were first pretreated with Bupivacaine, then 1 day later immunized either with pARC-gB or pARC-pp65 (80 µg/mouse). Mice were boosted 8 days later with DNA. Eight days after the second DNA dose mice were i.n. challenged
20 either with 5×10^6 pfu of Vaccinia WR-gB or Vaccinia WR-pp65. Lungs were taken at the time of virus challenge (day 0) and at days 1, 3, 4, 5, and 7 after challenge for virus titration. Lungs were homogenized, freeze-thaw 3
25 titration.

Table 5

Virus titers in the lungs of BALB/c mice immunized with pΔRC-gB or pΔRC-pp65 and challenged i.n. with Vac-gB

5	days after challenge	<u>Vac-gB titer (log±SD) in lungs*</u>		
		pΔRC-gB- immunized	pΔRC-pp65- immunized	Diff. in titer (log)
	0	3.29±2.83	3.29±2.83	0
10	1	2.24±2.9	2.76±2.51	-0.25
	3	4.86±4.61	5.60±5.45	0.53
	4	4.54±4.47	5.24±4.9	1.13
	5	4.33±3.82	5.03±4.9	1.43
	7	2.85±2.84	4.17±4.27	1.04
15	*Mean of titer (log) ± SD of 3 or 4 mice			

Table 6

Virus titers in the lungs of BALB/c mice immunized with pΔRC-gB or pΔRC-pp65 and challenged i.n. with Vac-pp65

20	days after challenge	<u>Vac - pp 65 titer (log±SD) in lungs*</u>	
		pΔRC-gB- immunized	pΔRC-pp65- immunized
25	0	5.52±4.83	5.52±4.83
	1	4.31±4.3	4.56±3.5
	3	7.68±6.75	7.15±7.11
	4	7.7±7.66	6.57±6.56
	5	7.45±6.79	6.02±6.14
30	7	7.17±6.17	6.23±6.08
*Mean of titer (log) ± SD of 3 or 4 mice			

This data demonstrate that immunization with either plasmid reduced the titer of the corresponding challenge virus by 0.5-1.4 log on days 3, 4, 5 and 7 after the challenge.

5 Example 12 - Secreted form of gB is more potent immunogen than membrane-bound gB

 To test whether gB bound to the membranes of gB-expressing cells or truncated form of gB lacking the transmembrane region of the molecule (it is secreted from
10 the cell) induce stronger immune responses, mice were immunized with p Δ RC-gB (expressing membrane-bound gB) or with p Δ RCgB₆₈₀ (expressing the secreted form of gB) and ELISA and neutralizing antibody responses were evaluated as follows.

15 Plasmids p Δ RC-gB (expressing the whole gB) and Δ RC-gB₆₈₀ (expressing N-terminal 680 amino acids of the gB molecule and lacking the transmembrane region) were used in the following immunization protocol. Groups of 10 mice (BALB/c, female, 8 weeks old, purchased from HSD),
20 were inoculated i.m. in the left leg with 50 μ g plasmid DNA/mouse/inoculation. Mice were not inoculated with bupivacaine prior to DNA inoculation. Two months later a booster immunization was given (same dose, route).

 Sera were tested in the gB-specific ELISA assay
25 described above before the booster inoculation and 1 month after booster. The results are shown in Table 7, which shows the OD values of serum dilutions of 1:40 of individual mice. Preimmune serum samples of 40 mice were included. Cut off value: OD = 0.15.

Table 7

HCMV ELISA antibodies induced by plasmids expressing
membrane-bound or secreted form of gB

		OD of sera of mice immunized with					
		<u>pΔRC-gB</u>			<u>pΔRC-gB₆₈₀</u>		
		<u># of</u>	<u>before</u>	<u>after</u>	<u># of</u>	<u>before</u>	<u>after</u>
		<u>mouse</u>	<u>booster</u>	<u>booster</u>	<u>mouse</u>	<u>booster</u>	<u>booster</u>
10	1		0.31	0.55	1	0.83	>3.00
	2		0.09	0.10	2	0.52	>3.00
	3		0.09	0.13	3	1.65	>3.00
	4		0.06	0.08	4	0.06	0.09
	5		0.07	0.07	5	1.29	>3.00
15	6		0.04	0.04	6	1.92	>3.00
	7		0.08	0.17	7	2.31	>3.00
	8		0.51	1.88	8	1.22	>3.00
	9		0.07	0.07	9	0.62	>3.00
	10		0.06	0.06	10	1.50	>3.00

20 The results in Table 7 show that ten mice immunized with the pΔRC-gB₆₈₀ were positive for stronger gB-specific antibody responses than mice immunized with pΔRC-gB.

25 Table 8 provides the results following the immunization protocol above, where the mice had been boosted after 2 months using the same protocol as described for the first immunization. Sera obtained 1 and 2 month after the booster were tested in a HCMV-microneutralization assay. Preimmune sera were included
30 as negative controls, NA titers ≥ 12 are considered positive.

Table 8

pΔRC-gB₆₈₀ expressing secreted form of gB induce stronger neutralizing antibody responses than pΔRC-gB expressing membrane-bound gB

NA titers of sera of mice 1 and 2 month after booster immunized with				
<u>pΔRC-gB</u>		<u>pΔRC-gB680</u>		
	1M	2M	1M	2M
10	16	24	128	64
	8	<8	64	32
	4	<4	256	192
	4	8	<4	12
	8	4	128	96
15	4	4	64	64
	8	24	64	32
	48	48	48	ND
	6	4	96	96
20	<6	4	16	24

As shown in Table 8, nine of the pΔRC-gB₆₈₀-immunized mice developed gB-specific antibodies, but only 3 of 10 responded in the pΔRC-gB-immunized group. HCMV-neutralizing antibody titers were also higher in the pΔRC-gB₆₈₀-immunized mice, 9 of 10 developed significant NA responses versus 3 of 10 in the pΔRC-gB-immunized group (Table 8).

These data show that the pΔRC-gB₆₈₀ plasmid expressing the N-terminal 680 amino acids of gB (lacking the transmembrane region of the protein) given intramuscularly induces more potent antibody responses to gB than the pΔRC-gB plasmid expressing the full gB.

Example 13 - pΔRC-gB₆₈₀ mixed with pΔRC-pp65 and given at one site or inoculated separately induce both gB- and pp65-specific antibodies

As shown above, pTet-gB and pΔRC-pp65 plasmids mixed
5 and inoculated at one site induced immune responses to both gB and pp65 indicating that there is no antigenic competition between gB and pp65. In this experiment whether the pΔRC-gB₆₈₀ (expressing the secreted form of gB) is suitable for immunization in a mixture with pΔRC-
10 pp65 was tested.

Groups of 10 BALB/c mice (female, HSD, 9-10 weeks old) were inoculated either with a mixture of two plasmids containing 50 μg of each in 200 μl: 100 μl (50 μg) into the left leg, 100 μl (50 μg) into the right leg;
15 or the two different plasmids were inoculated separately: one kind of DNA (100 μl/50 μg) into the left leg, the other kind of plasmid (100 μl/50 μg) into the right leg. A booster immunization was given 1 month later. The plasmids used in this study were pΔRC-pp65, pΔRC-gB, and
20 pΔRC-gB₆₈₀. Table 9 shows results obtained with sera taken 8 days after booster. The ELISA antigen was purified gB. Cut off value: 0.081.

The results show that mice immunized with mixtures of pΔRC-gB and pΔRC-pp65 developed both gB and pp65 ELISA
25 antibodies. Similar responses were observed in mice immunized with the two plasmids given at separate sites (Table 10 below). HCMV-gB-specific antibody responses in mice immunized with pΔRC-gB₆₈₀ either given in mixture with pΔRC-pp65 or at separate sites were stronger than in
30 mice immunized with the full-gB-expressing pΔRC-gB (these results confirm that the secreted form of gB is a stronger immunogen than the membrane-bound form).

Table 9

pΔRC-gB₆₈₀ mixed with pΔRC-pp65 and given at one site or inoculated separately induce gB-specific antibodies

5 gB-specific antibody (OD at serum dilutions of 1:40)								
mice inoculated with pΔRC-gB and pΔRC-pp65					mice inoculated with pΔRC-gB ₆₈₀ and pΔRC-pp65			
10	at one		at two		at one		at two	
	mouse	site	mouse	sites	mouse	site	mouse	sites
	#326	0.085	#356	0.115	#341	1.280	#336	1.058
	#327	0.193	#357	0.082	#342	1.070	#337	0.550
	#328	0.121	#358	0.099	#343	1.385	#338	0.193
	#329	0.060	#359	0.107	#344	1.190	#339	1.039
15	#330	0.115	#360	0.107	#345	2.588	#340	0.207
	#331	0.093	#361	NT	#351	1.037	#346	0.288
	#332	0.061	#362	0.092	#352	0.771	#347	0.220
	#333	0.089	#363	0.065	#353	0.493	#348	0.513
	#334	0.078	#364	0.152	#354	0.560	#349	0.223
20	#335	0.088	#365	0.082	#355	0.933	#350	0.719
	Mean	0.098		0.100		1.130		0.521
	OD:							

25 Mice immunized as above with the mixture of pΔRC-gB₆₈₀ and pΔRC-pp65 showed gB-specific antibody responses similar to those observed in mice immunized with the two kinds of plasmids given at separate sites. Results of pp65-specific antibody responses showed that mice responded to the pp65 antigen regardless of immunization with a mixture or with plasmids given at separate sites (Table 10). Table 10 shows results obtained with sera taken 8 days after booster (cut off value: 0.050).

Table 10

pΔRC-gB₆₈₀ mixed with pΔRC-pp65 and given at one site
or inoculated separately induce pp65-specific antibodies

5	pp65-specific antibody (OD at serum dilutions of 1:40)							
	mice inoculated with pΔRC-gB and pΔRC-pp65 pp65				mice inoculated with pΔRC-gB ₆₈₀ and pΔRC- pp65			
10	<u>mouse</u>	<u>at one site</u>	<u>mouse</u>	<u>at two sites</u>	<u>mouse</u>	<u>at one site</u>	<u>mouse</u>	<u>at two sites</u>
	#326	0.037	#356	0.000	#341	0.389	#336	0.276
	#327	0.149	#357	0.000	#342	0.238	#337	0.295
	#328	0.002	#358	0.508	#343	0.440	#338	0.000
15	#329	0.000	#359	0.008	#344	0.077	#339	0.009
	#330	0.009	#360	0.176	#345	0.008	#340	0.030
	#331	0.007	#361	dead	#351	0.081	#346	0.051
	#332	0.014	#362	0.009	#352	0.077	#347	0.124
	#333	0.000	#363	0.028	#353	0.049	#348	0.281
20	#334	0.000	#364	0.097	#354	0.016	#349	0.118
	#335	0.008	#365	0.201	#355	0.178	#350	0.014
	Mean	0.014		0.109		0.154		0.111
	OD:							
25	The data show that mice develop significant immune responses both to gB and pp65 after immunization with a mixture of pΔRC-gB ₆₈₀ and pΔRC-pp65, indicating that these two HCMV antigens are able to induce parallel immune responses when introduced by expression plasmids							
30	to the immune system.							

Example 14 - Immunization Studies in Mice Immunized with HCMV Plasmid Vectors Expressing Full-Length and Transmembrane-Deleted gB

As shown in the studies described above, full-length
5 gB and transmembrane-deleted gB have been found to induce
a strong and long-term antibody response when delivered
by plasmid DNA. The following experiments provide
further evidence of this effect.

A. pCBgB and pCBgB Δ tm

10 The gB open reading frame (ORF, nucleotides 1-
2724) was obtained from the CMV Towne strain [SEQ ID NO:
1] using conventional techniques. The gB Δ tm
(transmembrane-deleted gB) was obtained from the wild
type gene by deleting in frame the sequences coding for
15 the hydrophobic transmembrane domain of the protein
[nucleotides 2143 - 2316 were deleted from the gB ORF,
SEQ ID NO:1]. These two coding sequences were
introduced into the polylinker of the eukaryotic
expression vector pCB11 corresponding to a commercially
20 available pUC backbone with the HCMV IE1
promoter/enhancer sequences and the terminator sequences
from the bovine growth hormone gene (Fig. 7A). The
resulting plasmids, pCBgB and pCBgB Δ tm expressing the
full-length gB and its truncated version, respectively,
25 are shown in Fig. 8. Protein expression from pCBgB and
from pCBgB Δ tm was confirmed by immunofluorescence and
immunoprecipitation after transfection into cultured CHO-
K1 cells. The immunoprecipitation experiment indicated
that only pCBgB Δ tm gave rise to a secreted form of gB
30 which could be recovered from the cell culture medium.

B. Immunization

The study described below was performed with
pCBgB and pCBgB Δ tm in 6-8 week old female BALB/c mice.
Anesthetized (xylazine + ketamine) mice (8 per group)
35 received three administrations of 50 μ g pCBgB or pCBgB Δ tm

at three week intervals (days 0, 21 and 42) either intramuscularly (IM) or intradermally (ID). For IM administration, DNA in 50 μ l of saline was injected into the quadriceps with a Hamilton syringe equipped with a 20 gauge needle. For ID administration, DNA in a total volume of 100 μ l of saline was injected into 5 sites of shaved dorsal skin with a pneumatic jet injector.

In each group, mice were labeled and bled on days 14 (following 1 injection), 35 (following 2 injections), 56 (following 3 injections), 116 and 202. The anti-urease IgG antibody response was followed by ELISA against recombinant gB produced in MRC5 cells infected with ALVAC-gB. The sera collected on days 116 and 202 were analyzed for HCMV neutralization in complement dependent microneutralization assay [Gonczol et al, cited above (1986)]. The data is provided in Table 11 and summarized in Fig. 9.

TABLE 11

INDIVIDUAL ELISA TITERS
IN MICE IMMUNIZED WITH HCMV GB PLASMID VECTORS

Day	# Mouse	Intramuscular		Intradermal		neg. serum
		pCBgB ELISA	pCBgBAtm ELISA	pCBgB ELISA	pCBgBAtm ELISA	
25	14	1	50	50	<50	<50
		2	<50	200	<50	<50
		3	100	9600	100	<50
		4	<50	300	<50	<50
		5	100	100	<50	<50
		6	<50	75	<50	50
30		7	100	75	<50	<50
		8	50	<50	<50	<50
35		1	100	100	75	50
		2	150	900	150	600
		3	200	12800	6400	2400
		4	150	3200	1600	200
		5	400	1200	100	1600
		6	100	1200	1200	6400
40		7	150	300	75	100
		8	150	100	200	150

TABLE 11 (con't)

INDIVIDUAL ELISA TITERS
IN MICE IMMUNIZED WITH HCMV GB PLASMID VECTORS

5	Day	# Mouse	Intramuscular		Intradermal		neg. serum ELISA
			pCBgB ELISA	pCBgBΔtm ELISA	pCBgB ELISA	pCBgBΔtm ELISA	
10	56	1	150	1600	200	1200	<50
		2	200	2400	200	38400	<50
		3	200	38400	6400	12800	
		4	75	61200	6400	12800	
		5	400	2400	1200	4800	
		6	100	38400	3200	9600	
		7	200	19200	600	1600	
		8	600	4800	1200	4800	
20	15	116	<50	1200	75	600	<50
		2	1600	800	37.5	12800	<50
		3	400	9600	1200	640	
		4	<50	25600	2400	4800	
		5	25	1600	150	800	
		6	<50	25600	1600	4800	
		7	<50	6400	300	800	
		8	200	1200	200	800	
30	25	202	<50	1000	50	250	<50
		2	400	1000	25	8000	<50
		3	1600	8000	800	3000	
		4	<50	64000	1600	1500	
		5	25	1500	50	500	
		6	<50	24000	1200	3000	
		7	<50	4000	200	375	
		8		1000	150	375	

As illustrated in Table 11 above and in Fig. 9, pCBgB and pCBgBΔtm plasmids induced serum IgGs against recombinant gB protein after IM or ID administration in BALB/c mice [pCBgBΔtm/ID ≥ pCBgBΔtm/IM >> pCBgB/ID ≥ pCBgB/IM]. pCBgB and pCBgBΔtm plasmids induced detectable neutralizing antibodies to HCMV (in vitro assay) after IM or ID administration in BALB/c mice [pCBgBΔtm > pCBgB].

pCB-gB and pCB-gB Δ tm have been observed to induce a strong and long-term antibody response. pCBgB and especially pCB-gB Δ tm induce neutralizing antibodies.

5 The nature of the response (IgG₁/IgG_{2a}) differs between pCB-gB and pCB-gB Δ tm. Particularly, pCB-gB has been observed to induce an IgG₁ (T_{H2}) response which is approximately equivalent to the IgG_{2a} (T_{H1}) response induced. In contrast, pCB-gB Δ tm has been observed to induce an IgG₁ response that is significantly stronger
10 that the IgG_{2a} response induced.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to
15 the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

International Application No: PCT/

/

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 8, lines 9-10 of the description ***A. IDENTIFICATION OF DEPOSIT ***

pTet-gB

Further deposits are identified on an additional sheet ☐ *

Name of depositary institution *

American Type Culture Collection

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, Maryland 20852 USA

Date of deposit *

April 23, 1996

Accession Number *

98029

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)PAUL F. URRUTIA *PU*

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is *

was

(Authorized Officer)

WHAT IS CLAIMED IS:

1. A DNA molecule which is non-replicating in mammals and comprises a sequence encoding a human cytomegalovirus antigen,
wherein the sequence is operably linked to regulatory sequences for expressing the antigen in mammals and wherein the antigen elicits an immune response in the mammal.
2. The DNA molecule according to claim 1 which is a plasmid.
3. The DNA molecule according to claim 1 or claim 2 wherein said antigen is selected from the group consisting of:
 - (a) gB;
 - (b) a gB derivative lacking at least the transmembrane domain;
 - (c) pp65;
 - (d) pp150;
 - (e) immediate-early exon-4; and
 - (f) combinations of (a) - (e).
4. The DNA molecule according to claim 3 which comprises a sequence encoding the gB and the pp65 antigens.
5. The DNA molecule according to claim 3 which comprises a sequence encoding the gB derivative and a sequence encoding the pp65 antigen.

6. A pTet-gB DNA plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gB gene and a tetracycline regulatable HCMV-immediate early promoter, said promoter controlling the expression of gB.

7. A pARC/CMV DNA plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gB gene and capable of expressing gB.

8. A pARC-gB₆₈₀ plasmid, said plasmid comprising the portion of the human cytomegalovirus (HCMV) gene encoding the N-terminal 680 amino acids of the gB protein (gB₁₋₆₈₀) and capable of expressing gB₁₋₆₈₀.

9. A pARC-pp65 plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gene encoding the HCMV pp65 tegument protein and capable of expressing pp65.

10. A pARC-pp150 plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gene encoding the HCMV pp150 tegument protein and capable of expressing pp150.

11. A pARC-exon-4 plasmid, said plasmid comprising the portion of the human cytomegalovirus (HCMV) gene encoding HCMV immediate-early (IE)-exon-4 and capable of expressing IE-exon-4.

12. An immunogenic composition comprising a carrier and a DNA molecule according to any of claims 1-5.

13. The immunogenic composition according to claim 12 wherein the DNA molecule is selected from the group consisting of:

- (a) pΔRC-gB;
- (b) pTet-gB;
- (c) pΔRC-pp65;
- (d) pΔRC-gB₆₈₀;
- (e) pΔRC-pp150; and
- (f) pΔRC-exon-4.

14. The immunogenic composition according to claim 12 or 13 comprising two or more DNA molecules.

15. The immunogenic composition according to claim 14 comprising a first DNA molecule which comprises a sequence encoding the gB antigen or a gB derivative, and a second DNA molecule which comprises a sequence encoding the pp65 antigen.

16. The immunogenic composition according to any of claims 12 to 15 wherein the carrier is selected from the group consisting of saline and isotonic water.

17. A method of inducing human cytomegalovirus-specific (HCMV) immune responses in an animal, comprising the step of administering to said animal an effective amount of a first immunogenic composition according to any of claims 12 to 16.

18. The method according to claim 17 wherein the composition comprises pTet-gB and pΔRC-pp65.

19. The method according to claim 16 further comprising the step of administering a second immunogenic composition to said animal, said second immunogenic composition comprising a plasmid selected from the group consisting of:

- (a) p Δ RC-gB;
- (b) pTet-gB;
- (c) p Δ RC-pp65;
- (d) p Δ RC-gB₆₈₀;
- (e) p Δ RC-pp150; and
- (f) p Δ RC-IE-Exon-4.

20. The method according to claim 17, wherein said second immunogenic composition is administered between about 2 to about 15 weeks following administration of said first immunogenic composition.

21. The use of a DNA molecule according to any of claims 1 to 5 or a plasmid according to any of claims 6 to 11 in the preparation of a medicament to treat a cytomegalovirus infection.

22. A method of priming immune responses to a selected human cytomegalovirus immunogenic composition, comprising the steps of:

administering a first immunogenic composition according to any of claims 12 to 16 and administering the selected human cytomegalovirus immunogenic composition.

23. The method according to claim 22 wherein the first immunogenic composition is administered between about 4 and 15 weeks prior to administration of the selected immunogenic composition.

24. The method according to claim 22 or claim 23 wherein the first immunogenic composition comprises pTet-gB.

25. The method according to claim 24, wherein pTet-gB is administered in an amount between about 50 μ g to about 160 μ g.

26. The method according to claim 22, wherein the selected immunogenic composition comprises an immunogen selected from the group consisting of a recombinant virus comprising an HCMV immunogen, an HCMV protein, and HCMV virions.

27. The method according to claim 26 wherein the HCMV protein is gB.

28. The method according to claim 26 wherein the recombinant virus is selected from the group consisting of Ad5.gb and Ad5-IE-exon-4.

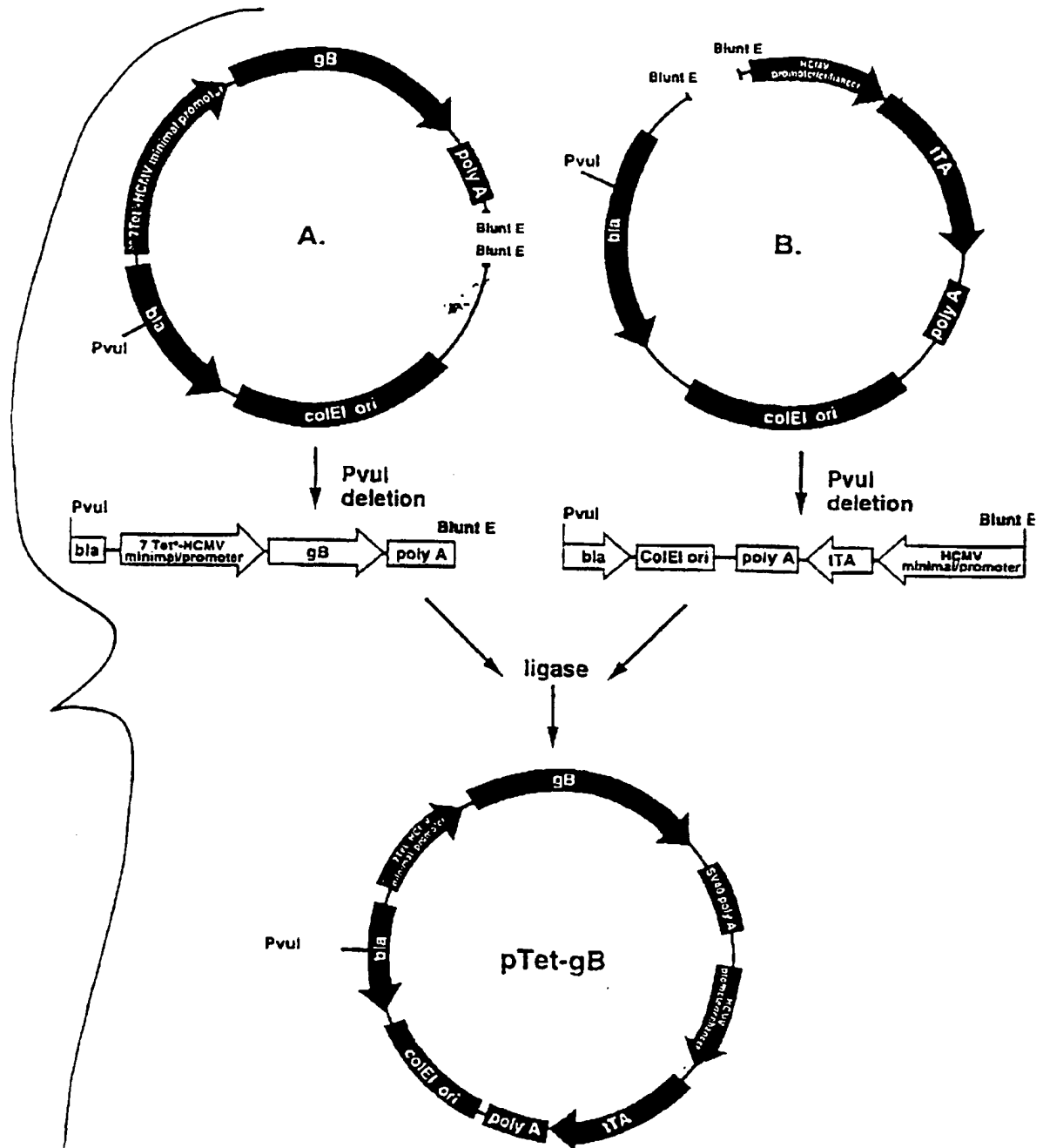


Fig. 1

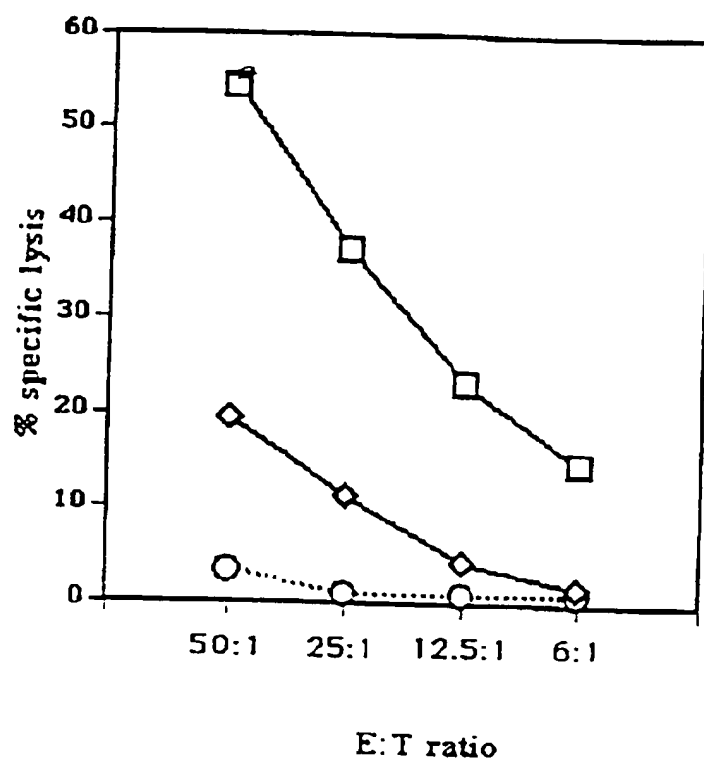


Fig. 2

FIGURE 3A

ATG	GAA	TCC	AGG	ATC	TGG	TGC	CTG	GTA	GTC	TGC	GTT	AAC	TTG	TGT	45
Met	Glu	Ser	Arg	Ile	Trp	Cys	Leu	Val	Val	Cys	Val	Asn	Leu	Cys	
1				5					10					15	
ATC	GTC	TGT	CTG	GGT	GCT	GCG	GTT	TCC	TCA	TCT	TCT	ACT	CGT	GGA	90
Ile	Val	Cys	Leu	Gly	Ala	Ala	Val	Ser	Ser	Ser	Ser	Thr	Arg	Gly	
				20					25					30	
ACT	TCT	GCT	ACT	CAC	AGT	CAC	CAT	TCC	TCT	CAT	ACG	ACG	TCT	GCT	135
Thr	Ser	Ala	Thr	His	Ser	His	His	Ser	Ser	His	Thr	Thr	Ser	Ala	
				35					40					45	
GCT	CAT	TCT	CGA	TCC	GGT	TCA	GTC	TCT	CAA	CGC	GTA	ACT	TCT	TCC	180
Ala	His	Ser	Arg	Ser	Gly	Ser	Val	Ser	Gln	Arg	Val	Thr	Ser	Ser	
				50					55					60	
CAA	ACG	GTC	AGC	CAT	GGT	GTT	AAC	GAG	ACC	ATC	TAC	AAC	ACT	ACC	225
Gln	Thr	Val	Ser	His	Gly	Val	Asn	Glu	Thr	Ile	Tyr	Asn	Thr	Thr	
				65					70					75	
CTC	AAG	TAC	GGA	GAT	GTG	GTG	GGG	GTC	AAC	ACC	ACC	AAG	TAC	CCC	270
Leu	Lys	Tyr	Gly	Asp	Val	Val	Gly	Val	Asn	Thr	Thr	Lys	Tyr	Pro	
				80					85					90	
TAT	CGC	GTG	TGT	TCT	ATG	GCA	CAG	GGT	ACG	GAT	CTT	ATT	CGC	TTT	315
Tyr	Arg	Val	Cys	Ser	Met	Ala	Gln	Gly	Thr	Asp	Leu	Ile	Arg	Phe	
				95					100					105	
GAA	CGT	AAT	ATC	GTC	TGC	ACC	TCG	ATG	AAG	CCC	ATC	AAT	GAA	GAC	360
Glu	Arg	Asn	Ile	Val	Cys	Thr	Ser	Met	Lys	Pro	Ile	Asn	Glu	Asp	
				110					115					120	
CTG	GAC	GAG	GGC	ATC	ATG	GTG	GTC	TAC	AAA	CGC	AAC	ATC	GTC	GCG	405
Leu	Asp	Glu	Gly	Ile	Met	Val	Val	Tyr	Lys	Arg	Asn	Ile	Val	Ala	
				125					130					135	
CAC	ACC	TTT	AAG	GTA	CGA	GTC	TAC	CAG	AAG	GTT	TTG	ACG	TTT	CGT	450
His	Thr	Phe	Lys	Val	Arg	Val	Tyr	Gln	Lys	Val	Leu	Thr	Phe	Arg	
				140					145					150	
CGT	AGC	TAC	GCT	TAC	ATC	CAC	ACC	ACT	TAT	CTG	CTG	GGC	AGC	AAC	495
Arg	Ser	Tyr	Ala	Tyr	Ile	His	Thr	Thr	Tyr	Leu	Leu	Gly	Ser	Asn	
				155					160					165	
ACG	GAA	TAC	GTG	GCG	CCT	CCT	ATG	TGG	GAG	ATT	CAT	CAT	ATC	AAC	540
Thr	Glu	Tyr	Val	Ala	Pro	Pro	Met	Trp	Glu	Ile	His	His	Ile	Asn	
				170					175					180	
AGT	CAC	AGT	CAG	TGC	TAC	AGT	TCC	TAC	AGC	CGC	GTT	ATA	GCA	GGC	585
Ser	His	Ser	Gln	Cys	Tyr	Ser	Ser	Tyr	Ser	Arg	Val	Ile	Ala	Gly	
				185					190					195	
ACG	GTT	TTC	GTG	GCT	TAT	CAT	AGG	GAC	AGC	TAT	GAA	AAC	AAA	ACC	630
Thr	Val	Phe	Val	Ala	Tyr	His	Arg	Asp	Ser	Tyr	Glu	Asn	Lys	Thr	
				200					205					210	

FIGURE 3B

ATG	CAA	TTA	ATG	CCC	GAC	GAT	TAT	TCC	AAC	ACC	CAC	AGT	ACC	CGT	675
Met	Gln	Leu	Met	Pro	Asp	Asp	Tyr	Ser	Asn	Thr	His	Ser	Thr	Arg	
				215					220					225	
TAC	GTG	ACG	GTC	AAG	GAT	CAA	TGG	CAC	AGC	CGC	GGC	AGC	ACC	TGG	720
Tyr	Val	Thr	Val	Lys	Asp	Gln	Trp	His	Ser	Arg	Gly	Ser	Thr	Trp	
				230					235					240	
CTC	TAT	CGT	GAG	ACC	TGT	AAT	CTG	AAT	TGT	ATG	GTG	ACC	ATC	ACT	765
Leu	Tyr	Arg	Glu	Thr	Cys	Asn	Leu	Asn	Cys	Met	Val	Thr	Ile	Thr	
				245					250					255	
ACT	GCG	CGC	TCC	AAG	TAT	CCC	TAT	CAT	TTT	TTC	GCA	ACT	TCC	ACG	810
Thr	Ala	Arg	Ser	Lys	Tyr	Pro	Tyr	His	Phe	Phe	Ala	Thr	Ser	Thr	
				260					265					270	
GGT	GAT	GTG	GTT	GAC	ATT	TCT	CCT	TTC	TAC	AAC	GGA	ACT	AAT	CGC	855
Gly	Asp	Val	Val	Asp	Ile	Ser	Pro	Phe	Tyr	Asn	Gly	Thr	Asn	Arg	
				275					280					285	
AAT	GCC	AGC	TAT	TTT	GGA	GAA	AAC	GCC	GAC	AAG	TTT	TTC	ATT	TTT	900
Asn	Ala	Ser	Tyr	Phe	Gly	Glu	Asn	Ala	Asp	Lys	Phe	Phe	Ile	Phe	
				290					295					300	
CCG	AAC	TAC	ACT	ATC	GTC	TCC	GAC	TTT	GGA	AGA	CCG	AAT	TCT	GCG	945
Pro	Asn	Tyr	Thr	Ile	Val	Ser	Asp	Phe	Gly	Arg	Pro	Asn	Ser	Ala	
				305					310					315	
TTA	GAG	ACC	CAC	AGG	TTG	GTG	GCT	TTT	CTT	GAA	CGT	GCG	GAC	TCA	990
Leu	Glu	Thr	His	Arg	Leu	Val	Ala	Phe	Leu	Glu	Arg	Ala	Asp	Ser	
				320					325					330	
GTG	ATC	TCC	TGG	GAT	ATA	CAG	GAC	GAG	AAG	AAT	GTT	ACT	TGT	CAA	1035
Val	Ile	Ser	Trp	Asp	Ile	Gln	Asp	Glu	Lys	Asn	Val	Thr	Cys	Gln	
				335					340					345	
CTC	ACT	TTC	TGG	GAA	GCC	TCG	GAA	CGC	ACC	ATT	CGT	TCC	GAA	GCC	1080
Leu	Thr	Phe	Trp	Glu	Ala	Ser	Glu	Arg	Thr	Ile	Arg	Ser	Glu	Ala	
				350					355					360	
GAG	GAC	TCG	TAT	CAC	TTT	TCT	TCT	GCC	AAA	ATG	ACC	GCC	ACT	TTC	1125
Glu	Asp	Ser	Tyr	His	Phe	Ser	Ser	Ala	Lys	Met	Thr	Ala	Thr	Phe	
				365					370					375	
TTA	TCT	AAG	AAG	CAA	GAG	GTG	AAC	ATG	TCC	GAC	TCT	GCG	CTG	GAC	1170
Leu	Ser	Lys	Lys	Gln	Glu	Val	Asn	Met	Ser	Asp	Ser	Ala	Leu	Asp	
				380					385					390	
TGT	GTA	CGT	GAT	GAG	GCC	ATA	AAT	AAG	TTA	CAG	CAG	ATT	TTC	AAT	1215
Cys	Val	Arg	Asp	Glu	Ala	Ile	Asn	Lys	Leu	Gln	Gln	Ile	Phe	Asn	
				395					400					405	
ACT	TCA	TAC	AAT	CAA	ACA	TAT	GAA	AAA	TAT	GGA	AAC	GTG	TCC	GTC	1260
Thr	Ser	Tyr	Asn	Gln	Thr	Tyr	Glu	Lys	Tyr	Gly	Asn	Val	Ser	Val	
				410					415					420	

FIGURE 3C

TTT	GAA	ACC	ACT	GGT	GGT	TTG	GTG	GTG	TTC	TGG	CAA	GGT	ATC	AAG	1305
Phe	Glu	Thr	Thr	Gly	Gly	Leu	Val	Val	Phe	Trp	Gln	Gly	Ile	Lys	
				425					430					435	
CAA	AAA	TCT	CTG	GTG	GAA	CTC	GAA	CGT	TTG	GCC	AAC	CGC	TCC	AGT	1350
Gln	Lys	Ser	Leu	Val	Glu	Leu	Glu	Arg	Leu	Ala	Asn	Arg	Ser	Ser	
				440					445					450	
CTG	AAT	CTT	ACT	CAT	AAT	AGA	ACC	AAA	AGA	AGT	ACA	GAT	GGC	AAC	1395
Leu	Asn	Leu	Thr	His	Asn	Arg	Thr	Lys	Arg	Ser	Thr	Asp	Gly	Asn	
				455					460					465	
AAT	GCA	ACT	CAT	TTA	TCC	AAC	ATG	GAG	TCG	GTG	CAC	AAT	CTG	GTC	1440
Asn	Ala	Thr	His	Leu	Ser	Asn	Met	Glu	Ser	Val	His	Asn	Leu	Val	
				470					475					480	
TAC	GCC	CAG	CTG	CAG	TTC	ACC	TAT	GAC	ACG	TTG	CGC	GGT	TAC	ATC	1485
Tyr	Ala	Gln	Leu	Gln	Phe	Thr	Tyr	Asp	Thr	Leu	Arg	Gly	Tyr	Ile	
				485					490					495	
AAC	CGG	GCG	CTG	GCG	CAA	ATC	GCA	GAA	GCC	TGG	TGT	GTG	GAT	CAA	1530
Asn	Arg	Ala	Leu	Ala	Gln	Ile	Ala	Glu	Ala	Trp	Cys	Val	Asp	Gln	
				500					505					510	
CGG	CGC	ACC	CTA	GAG	GTC	TTC	AAG	GAA	CTT	AGC	AAG	ATC	AAC	CCG	1575
Arg	Arg	Thr	Leu	Glu	Val	Phe	Lys	Glu	Leu	Ser	Lys	Ile	Asn	Pro	
				515					520					525	
TCA	GCT	ATT	CTC	TCG	GCC	ATC	TAC	AAC	AAA	CCG	ATT	GCC	GCG	CGT	1620
Ser	Ala	Ile	Leu	Ser	Ala	Ile	Tyr	Asn	Lys	Pro	Ile	Ala	Ala	Arg	
				530					535					540	
TTC	ATG	GGT	GAT	GTC	CTG	GGT	CTG	GCC	AGC	TGC	GTG	ACC	ATT	AAC	1665
Phe	Met	Gly	Asp	Val	Leu	Gly	Leu	Ala	Ser	Cys	Val	Thr	Ile	Asn	
				545					550					555	
CAA	ACC	AGC	GTC	AAG	GTG	CTG	CGT	GAT	ATG	AAT	GTG	AAG	GAA	TCG	1710
Gln	Thr	Ser	Val	Lys	Val	Leu	Arg	Asp	Met	Asn	Val	Lys	Glu	Ser	
				560					565					570	
CCA	GGA	CGC	TGC	TAC	TCA	CGA	CCA	GTG	GTC	ATC	TTT	AAT	TTC	GCC	1755
Pro	Gly	Arg	Cys	Tyr	Ser	Arg	Pro	Val	Val	Ile	Phe	Asn	Phe	Ala	
				575					580					585	
AAC	AGC	TCG	TAC	GTG	CAG	TAC	GGT	CAA	CTG	GGC	GAG	GAT	AAC	GAA	1800
Asn	Ser	Ser	Tyr	Val	Gln	Tyr	Gly	Gln	Leu	Gly	Glu	Asp	Asn	Glu	
				590					595					600	
ATC	CTG	TTG	GGC	AAC	CAC	CGC	ACT	GAG	GAA	TGT	CAG	CTT	CCC	AGC	1845
Ile	Leu	Leu	Gly	Asn	His	Arg	Thr	Glu	Glu	Cys	Gln	Leu	Pro	Ser	
				605					610					615	
CTC	AAG	ATC	TTC	ATC	GCC	GGC	AAC	TCG	GCC	TAC	GAG	TAC	GTG	GAC	1890
Leu	Lys	Ile	Phe	Ile	Ala	Gly	Asn	Ser	Ala	Tyr	Glu	Tyr	Val	Asp	
				620					625					630	

FIGURE 3D

TAC	CTC	TTC	AAA	CGC	ATG	ATT	GAC	CTC	AGC	AGC	ATC	TCC	ACC	GTC	1935
Tyr	Leu	Phe	Lys	Arg	Met	Ile	Asp	Leu	Ser	Ser	Ile	Ser	Thr	Val	
				635					640					645	
GAC	AGC	ATG	ATC	GCC	CTA	GAC	ATC	GAC	CCG	CTG	GAA	AAC	ACC	GAC	1980
Asp	Ser	Met	Ile	Ala	Leu	Asp	Ile	Asp	Pro	Leu	Glu	Asn	Thr	Asp	
				650					655					660	
TTC	AGG	GTA	CTG	GAA	CTT	TAC	TCG	CAG	AAA	GAA	TTG	CGT	TCC	AGC	2025
Phe	Arg	Val	Leu	Glu	Leu	Tyr	Ser	Gln	Lys	Glu	Leu	Arg	Ser	Ser	
				665					670					675	
AAC	GTT	TTT	GAT	CTC	GAG	GAG	ATC	ATG	CGC	GAG	TTC	AAT	TCG	TAT	2070
Asn	Val	Phe	Asp	Leu	Glu	Glu	Ile	Met	Arg	Glu	Phe	Asn	Ser	Tyr	
				680					685					690	
AAG	CAG	CGG	GTA	AAG	TAC	GTG	GAG	GAC	AAG	GTA	GTC	GAC	CCG	CTG	2115
Lys	Gln	Arg	Val	Lys	Tyr	Val	Glu	Asp	Lys	Val	Val	Asp	Pro	Leu	
				695					700					705	
CCG	CCC	TAC	CTC	AAG	GGT	CTG	GAC	GAC	CTC	ATG	AGC	GGC	CTG	GGC	2160
Pro	Pro	Tyr	Leu	Lys	Gly	Leu	Asp	Asp	Leu	Met	Ser	Gly	Leu	Gly	
				710					715					720	
GCC	GCG	GGA	AAG	GCC	GTT	GGC	GTA	GCC	ATT	GGG	GCC	GTG	GGT	GGC	2205
Ala	Ala	Gly	Lys	Ala	Val	Gly	Val	Ala	Ile	Gly	Ala	Val	Gly	Gly	
				725					730					735	
GCG	GTG	GCC	TCC	GTG	GTC	GAA	GGC	GTT	GCC	ACC	TTC	CTC	AAA	AAC	2250
Ala	Val	Ala	Ser	Val	Val	Glu	Gly	Val	Ala	Thr	Phe	Leu	Lys	Asn	
				740					745					750	
CCC	TTC	GGA	GCC	TTC	ACC	ATC	ATC	CTC	GTG	GCC	ATA	GCC	GTC	GTC	2295
Pro	Phe	Gly	Ala	Phe	Thr	Ile	Ile	Leu	Val	Ala	Ile	Ala	Val	Val	
				755					760					765	
ATT	ATC	ATT	TAT	TTG	ATC	TAT	ACT	CGA	CAG	CGG	CGT	CTC	TGC	ATG	2340
Tyr	Leu	Ile	Tyr	Thr	Arg	Gln	Arg	Arg	Leu	Cys	Met	Gln	Pro	Leu	
				770					775					780	
CAG	CCG	CTG	CAG	AAC	CTC	TTT	CCC	TAT	CTG	GTG	TCC	GCC	GAC	GGG	2385
Ile	Ile	Ile	Gln	Asn	Leu	Phe	Pro	Tyr	Leu	Val	Ser	Ala	Asp	Gly	
				785					790					795	
ACC	ACC	GTG	ACG	TCG	GGC	AAC	ACC	AAA	GAC	ACG	TCG	TTA	CAG	GCT	2430
Thr	Thr	Val	Thr	Ser	Gly	Asn	Thr	Lys	Asp	Thr	Ser	Leu	Gln	Ala	
				800					805					810	
CCG	CCT	TCC	TAC	GAG	GAA	AGT	GTT	TAT	AAT	TCT	GGT	CGC	AAA	GGA	2475
Pro	Pro	Ser	Tyr	Glu	Glu	Ser	Val	Tyr	Asn	Ser	Gly	Arg	Lys	Gly	
				815					820					825	
CCG	GGA	CCA	CCG	TCG	TCT	GAT	GCA	TCC	ACG	GCG	GCT	CCG	CCT	TAC	2520
Pro	Gly	Pro	Pro	Ser	Ser	Asp	Ala	Ser	Thr	Ala	Ala	Pro	Pro	Tyr	
				830					835					840	

FIGURE 3E

ACC	AAC	GAG	CAG	GCT	TAC	CAG	ATG	CTT	CTG	GCC	CTG	GTC	CGT	CTG	2565
Thr	Asn	Glu	Gln	Ala	Tyr	Gln	Met	Leu	Leu	Ala	Leu	Val	Arg	Leu	
				845					850					855	
GAC	GCA	GAG	CAG	CGA	GCG	CAG	CAG	AAC	GGT	ACA	GAT	TCT	TTG	GAC	2610
Asp	Ala	Glu	Gln	Arg	Ala	Gln	Gln	Asn	Gly	Thr	Asp	Ser	Leu	Asp	
				860					865					870	
GGA	CAG	ACT	GGC	ACG	CAG	GAC	AAG	GGA	CAG	AAG	CCC	AAC	CTG	CTA	2655
Gly	Gln	Thr	Gly	Thr	Gln	Asp	Lys	Gly	Gln	Lys	Pro	Asn	Leu	Leu	
				875					880					885	
GAC	CGA	CTG	CGA	CAC	CGC	AAA	AAC	GGC	TAC	CGA	CAC	TTG	AAA	GAC	2700
Asp	Arg	Leu	Arg	His	Arg	Lys	Asn	Gly	Tyr	Arg	His	Leu	Lys	Asp	
				890					895					900	
TCC	GAC	GAA	GAA	GAG	AAC	GTC	TGA								2724
Ser	Asp	Glu	Glu	Glu	Asn	Val									
				905											

FIGURE 4A

ATG	AAA	CAG	ATT	AAG	GTT	CGA	GTG	GAC	ATG	CTG	CGG	CAT	AGA	ATC	45
Met	Lys	Gln	Ile	Lys	Val	Arg	Val	Asp	Met	Leu	Arg	His	Arg	Ile	
1				5					10					15	
AAG	GAG	CAC	ATG	CTG	AAA	AAA	TAT	ACC	CAG	ACG	GAA	GAG	AAA	TTC	90
Lys	Glu	His	Met	Leu	Lys	Lys	Tyr	Thr	Gln	Thr	Glu	Glu	Lys	Phe	
				20					25					30	
ACT	GGC	GCC	TTT	AAT	ATG	ATG	GGA	GGA	TGT	TTG	CAG	AAT	GCC	TTA	135
Thr	Gly	Ala	Phe	Asn	Met	Met	Gly	Gly	Cys	Leu	Gln	Asn	Ala	Leu	
				35					40					45	
GAT	ATC	TTA	GAT	AAG	GTT	CAT	GAG	CCT	TTC	GAG	GAG	ATG	AAG	TGT	180
Asp	Ile	Leu	Asp	Lys	Val	His	Glu	Pro	Phe	Glu	Glu	Met	Lys	Cys	
				50					55					60	
ATT	GGG	CTA	ACT	ATG	CAG	AGC	ATG	TAT	GAG	AAC	TAC	ATT	GTA	CCT	225
Ile	Gly	Leu	Thr	Met	Gln	Ser	Met	Tyr	Glu	Asn	Tyr	Ile	Val	Pro	
				65					70					75	
GAG	GAT	AAG	CGG	GAG	ATG	TGG	ATG	GCT	TGT	ATT	AAG	GAG	CTG	CAT	270
Glu	Asp	Lys	Arg	Glu	Met	Trp	Met	Ala	Cys	Ile	Lys	Glu	Leu	His	
				80					85					90	
GAT	GTG	AGC	AAG	GGC	GCC	GCT	AAC	AAG	TTG	GGG	GGT	GCA	CTG	CAG	315
Asp	Val	Ser	Lys	Gly	Ala	Ala	Asn	Lys	Leu	Gly	Gly	Ala	Leu	Gln	
				95					100					105	
GCT	AAG	GCC	CGT	GCT	AAA	AAG	GAT	GAA	CTT	AGG	AGA	AAG	ATG	ATG	360
Ala	Lys	Ala	Arg	Ala	Lys	Lys	Asp	Glu	Leu	Arg	Arg	Lys	Met	Met	
				110					115					120	
TAT	ATG	TGC	TAC	AGG	AAT	ATA	GAG	TTC	TTT	ACC	AAG	AAC	TCA	GCC	405
Tyr	Met	Cys	Tyr	Arg	Asn	Ile	Glu	Phe	Phe	Thr	Lys	Asn	Ser	Ala	
				125					130					135	
TTC	CCT	AAG	ACC	ACC	AAT	GGC	TGC	AGT	CAG	GCC	ATG	GCG	GCA	TTG	450
Phe	Pro	Lys	Thr	Thr	Asn	Gly	Cys	Ser	Gln	Ala	Met	Ala	Ala	Leu	
				140					145					150	
CAG	AAC	TTG	CCT	CAG	TGC	TCC	CCT	GAT	GAG	ATT	ATG	GCT	TAT	GCC	495
Gln	Asn	Leu	Pro	Gln	Cys	Ser	Pro	Asp	Glu	Ile	Met	Ala	Tyr	Ala	
				155					160					165	
CAG	AAA	ATA	TTT	AAG	ATT	TTG	GAT	GAG	GAG	AGA	GAC	AAG	GTG	CTC	540
Gln	Lys	Ile	Phe	Lys	Ile	Leu	Asp	Glu	Glu	Arg	Asp	Lys	Val	Leu	
				170					175					180	
ACG	CAC	ATT	GAT	CAC	ATA	TTT	ATG	GAT	ATC	CTC	ACT	ACA	TGT	GTG	585
Thr	His	Ile	Asp	His	Ile	Phe	Met	Asp	Ile	Leu	Thr	Thr	Cys	Val	
				185					190					195	
GAA	ACA	ATG	TGT	AAT	GAG	TAC	AAG	GTC	ACT	AGT	GAC	GCT	TGT	ATG	630
Glu	Thr	Met	Cys	Asn	Glu	Tyr	Lys	Val	Thr	Ser	Asp	Ala	Cys	Met	
				200					205					210	

FIGURE 4B

ATG ACC ATG TAC GGG GGC ATC TCT CTC TTA AGT GAG TTC TGT CGG	675
Met Thr Met Tyr Gly Gly Ile Ser Leu Leu Ser Glu Phe Cys Arg	215 220 225
GTG CTG TCC TGC TAT GTC TTA GAG GAG ACT AGT GTG ATG CTG GCC	720
Val Leu Ser Cys Tyr Val Leu Glu Glu Thr Ser Val Met Leu Ala	230 235 240
AAG CGG CCT CTG ATA ACC AAG CCT GAG GTT ATC AGT GTA ATG AAG	765
Lys Arg Pro Leu Ile Thr Lys Pro Glu Val Ile Ser Val Met Lys	245 250 255
CGC CGC ATT GAG GAG ATC TGC ATG AAG GTC TTT GCC CAG TAC ATT	810
Arg Arg Ile Glu Glu Ile Cys Met Lys Val Phe Ala Gln Tyr Ile	260 265 270
CTG GGG GCC GAT CCT CTG AGA GTC TGC TCT CCT AGT GTG GAT GAC	855
Leu Gly Ala Asp Pro Leu Arg Val Cys Ser Pro Ser Val Asp Asp	275 280 285
CTA CGG GCC ATC GCC GAG GAG TCA GAT GAG GAA GAG GCT ATT GTA	900
Leu Arg Ala Ile Ala Glu Glu Ser Asp Glu Glu Glu Ala Ile Val	290 295 300
GCC TAC ACT TTG GCC ACC CGT GGT GCC AGC TCC TCT GAT TCT CTG	945
Ala Tyr Thr Leu Ala Thr Arg Gly Ala Ser Ser Ser Asp Ser Leu	305 310 315
GTG TCA CCC CCA GAG TCC CCT GTA CCC GCG ACT ATC CCT CTG TCC	990
Val Ser Pro Pro Glu Ser Pro Val Pro Ala Thr Ile Pro Leu Ser	320 325 330
TCA GTA ATT GTG GCT GAG AAC AGT GAT CAG GAA GAA AGT GAG CAG	1035
Ser Val Ile Val Ala Glu Asn Ser Asp Gln Glu Glu Ser Glu Gln	335 340 345
AGT GAT GAG GAA GAG GAG GAG GGT GCT CAG GAG GAG CGG GAG GAC	1080
Ser Asp Glu Glu Glu Glu Glu Gly Ala Gln Glu Glu Arg Glu Asp	350 355 360
ACT GTG TCT GTC AAG TCT GAG CCA GTG TCT GAG ATA GAG GAA GTT	1125
Thr Val Ser Val Lys Ser Glu Pro Val Ser Glu Ile Glu Glu Val	365 370 375
GCC CCA GAG GAA GAG GAG GAT GGT GCT GAG GAA CCC ACC GCC TCT	1170
Ala Pro Glu Glu Glu Glu Asp Gly Ala Glu Glu Pro Thr Ala Ser	380 385 390
GGA GGC AAG AGC ACC CAC CCT ATG GTG ACT AGA AGC AAG GCT GAC	1215
Gly Gly Lys Ser Thr His Pro Met Val Thr Arg Ser Lys Ala Asp	395 400 405
CAG TAA	1221
Gln	

FIGURE 5

Not linker

Met Ala Ser Val Leu Gly Pro Ile Ser Gly His Val Leu Lys Ala Val Phe Ser Arg Gly Asp Thr Pro Val Leu Pro His Glu Thr Arg
 CCC ATG GCA TCC GTA CTG GCT CCC ATT TCG CGC CAC CTC CTC AAA GGC CTC TTT ACT CGC CGC GAC ACC CCG CTC CTC CCC CAC GAC ACC CGA 93
 ATA
 Ile

Leu Leu Gln Thr Gly Ile His Val Arg Val Ser Gln Pro Ser Leu Ile Leu Val Ser Gln Tyr Thr Pro Asp Ser Thr Pro Cys His Arg Gly
 CTC CTC CAC ACC GCT ATC CAC CTC CGC CTG ACC CAC CCC TCG CTC ATC CTC CTC TCG CAC TAC ACC CGC CAC TCG ACC CGA TCC CAC CGC GGC 106
 A

Asp Asn Gln Leu Gln Val Gln His Thr Tyr Phe Thr Gly Ser Glu Val Glu Asn Val Ser Val Asn Val His Asn Pro Thr Gly Arg Ser Ile
 CAC AAT CAC CTC CAG CTC CAG CAC ACC TAC TTT ACC GGC ACC GAG CTC GAG AAC CTC TCG CTC AAC Val His Asn Pro Thr Gly Arg Ser Ile
 3' splice donor 279

Cys Pro Ser Gln Glu Pro Met Ser Ile Tyr Val Tyr Ala Leu Pro Leu Lys Met Leu Asn Ile Pro Ser Ile Asn Val His His Tyr Pro Ser
 TCC CCC ACC CAA GAG CCC ATG TCG ATC TAT CTC TAC TCC CTC CGC CTC AAC ATG CTC AAC ATC CCC ACC ATC AAC GTC CAC CAC TAC CCC TCC 372
 3' splice acceptor

Ala Ala Glu Arg Lys His Arg His Leu Pro Val Ala Asp Ala Val Ile His Ala Ser Gly Lys Gln Met Trp Gln Ala Arg Leu Thr Val Ser
 CGC GGC GAG CCC AAA CAC CGA CAC CTC CCC GTA GCT CAC GCT CTC ATT CAC GGC TCG GGC AAG CAG ATC TCC CAC GGC GCT CTC ACC CTC TCC 465

Gly Leu Ala Trp Thr Arg Gln Gln Asn Gln Trp Lys Gln Pro Asp Val Tyr Tyr Thr Ser Ala Phe Val Phe Pro Thr Lys Asp Val Ala Leu
 GGA CTC CCC TCC ACC GCT CAG CAG AAC CAG TCC AAA CAC CCC CAC CTC TAC TAC ACC TCA GGC TTC CTC TTT CCC ACC AAG CAC CTC CGA CTC 558

Arg His Val Val Cys Ala His Glu Leu Val Cys Ser Met Glu Asn Thr Arg Ala Thr Lys Met Gln Val Ile Gly Asp Gln Tyr Val Lys Val
 CGC CAC CTC CTC TCC GGC CAC CAC CTC GTT TCC TCC ATC CAC AAC ACC CGC GCA ACC AAC ATC CAC CTC ATA GCT CAC CAG TAC CTC AAG CTC 651

Tyr Leu Glu Ser Phe Cys Glu Asp Val Pro Ser Gly Lys Leu Phe Met His Val Thr Leu Gly Ser Asp Val Glu Glu Asp Leu Thr Met Thr
 TAC CTC GAG TCC TTC TCC CAC CAC CTC CTC TCC GGC AAG CTC TTT ATC CAC CTC ACC CTC GGC TCT CAC CTC GAA CAG CAC CTC ACC ATC ACC 744

Arg Asn Pro Gln Pro Phe Met Arg Pro His Glu Arg Asn Gly Phe Thr Val Leu Cys Pro Lys Asn Met Ile Ile Lys Pro Gly Lys Ile Ser
 CGC AAC CGC CAA CCC TTC ATC CCC CCC CAC CAG CGC AAC CCC TTT ACC CTC TTC TGT CCC AAA AAT ATC ATA ATC AAA CGC GGC AAG ATC TCC 837

His Ile Met Leu Asp Val Ala Phe Thr Ser His Glu His Phe Gly Leu Leu Cys Pro Lys Ser Ile Pro Gly Leu Ser Ile Ser Gly Asn Leu
 CAC ATC ATC CTC GAT CTC GCT TTT ACC TCA CAC CAC CAT TTT GGC CTC CTC TGT CCC AAG ACC ATC CCC GGC CTC ACC ATC TCA GCT AAC CTA 930
 C

Leu Met Asn Gly Gln Gln Ile Phe Leu Glu Val Gln Ala Ile Arg Glu Thr Val Glu Leu Arg Gln Tyr Asp Pro Val Ala Ala Leu Phe Phe
 TTG ATG AAC GGC CAC CAC ATC TTC CTC GAG CTC CAA GGC ATA GGC GAG ACC CTC GAA CTC GCT CAC TAC CAT CCC GTC GCT CGC CTC TTC TTT 1023
 A C

Phe Asp Ile Asp Leu Leu Leu Gln Arg Gly Pro Gln Tyr Ser Glu His Pro Thr Phe Thr Ser Gln Tyr Arg Ile Gln Gly Lys Leu Glu Tyr
 TTC CAT ATC CAC TTC CTC CTC CAC CGC CGC GCT CAC TAC AAC CAA CAC CCC ACC TTC ACC ACC CAC TAT CCC ATC CAG GGC AAG CTT CAG TAC 1116
 C

Arg His Thr Trp Asp Arg His Asp Glu Gly Ala Ala Gln Gly Asp Asp Asp Val Trp Thr Ser Gly Ser Asp Ser Asp Glu Glu Leu Val Thr
 CGA CAC ACC TCC GAC CGC CAC CAC CAC GCT CCC GCT CAC GGC CAC CAC CTC TCG ACC ACC CGA TCC CAC TCC CAC CAC CAA CTC GTA ACC 1209
 A

Thr Glu Arg Lys Thr Pro Asp Val Thr Gly Gly Gly Ala Met Ala Gly Ala Ser Thr Ser Ala Gly Arg Lys Arg Lys Ser Ala Ser Ser Ala
 ACC GAG CGC AAG ACC CCC GGC GTC ACC GGC GGC GGC CCC ATC GGC GGC GGC TTC ACT TCC CGC GGC GGC AAA CGC AAA TCA CGA TCC TCC CCC 1302
 C

Thr Ala Cys Thr Ala Gly Val Met Thr Arg Gly Arg Leu Lys Ala Gln Ser Thr Val Ala Pro Glu Glu Asp Thr Asp Glu Asp Ser Asp Asn
 ACC GGC TCC ACC GGC GCT ATC ACA GGC GGC GGC CTT AAG GGC CAC TCC ACC GTC CGC CCC GAA CAC CAC ACC GAC GAG CAT TCC CAC AAC 1395
 Ser

Glu Ile His Asp Pro Ala Val Phe Thr Trp Pro Pro Trp Gln Ala Gly Ile Leu Ala Arg Asn Leu Val Pro Met Val Ala Thr Val Gln Gly
 CAA ATC CAC AAT CGC CCC GTC TTC ACC TCC CGC CCC TCG CAG GGC GGC ATC CTC GCT CGC AAC CTC CTC CGC ATC GCT ACC CTT CAG GGT 1488

Gln Asn Leu Lys Tyr Gln Glu Phe Phe Trp Asp Ala Asn Asp Ile Tyr Arg Ile Phe Ala Glu Leu Glu Gly Val Trp Gln Pro Ala Ala Gln
 CAG AAT CTC AAG TAC CAG CAG TTC TTC TCC CAC GGC AAC CAC ATC TAC CCC ATC TTC CCC GAA TTG GAA GGC GTA TCG CAG CCC GCT CGC CAA 1581
 A

Pro Lys Arg Arg Arg His Arg Gln Asp Ala Leu Pro Gly Pro Cys Ile Ala Ser Thr Pro Lys Lys His Arg Gly STOP
 CCC AAA GGT CCC CCC CAC CGC CAA CAC CCC TTC CCC CGC CCA TCC ATC CCC TCG ACC CGC AAA AAG CAC CCA GCT TCA GCGACGCGCC GCGACGCGTT 1679
 A G

ACGACGACGCT TATAAAAACC CAGGTGCACT CAGACGCGC ACTTTTGGC GCGACGCTG TCGCGCTCC TATATTGCG ACAGTGGCG CAGCCTTCC CGACCTGCA 1789
 A

CGACGACGCG TTACGCTTG CGCATCGCT CAGCTGCGCC CTCATCGCG CTCGCGC ATC TCT CAC GCA TCC TCC TCG CCC GGT CAG CGA CCC TCC TCC CAA GGC 1894
 T C A

Ala Ala Ile Ser Glu Ala Glu Ala Ala Ser Gly Ser
 CCC GGC ATC ACC CAG CCC GAA GGC GGC ACC CGA ACC TT 1932

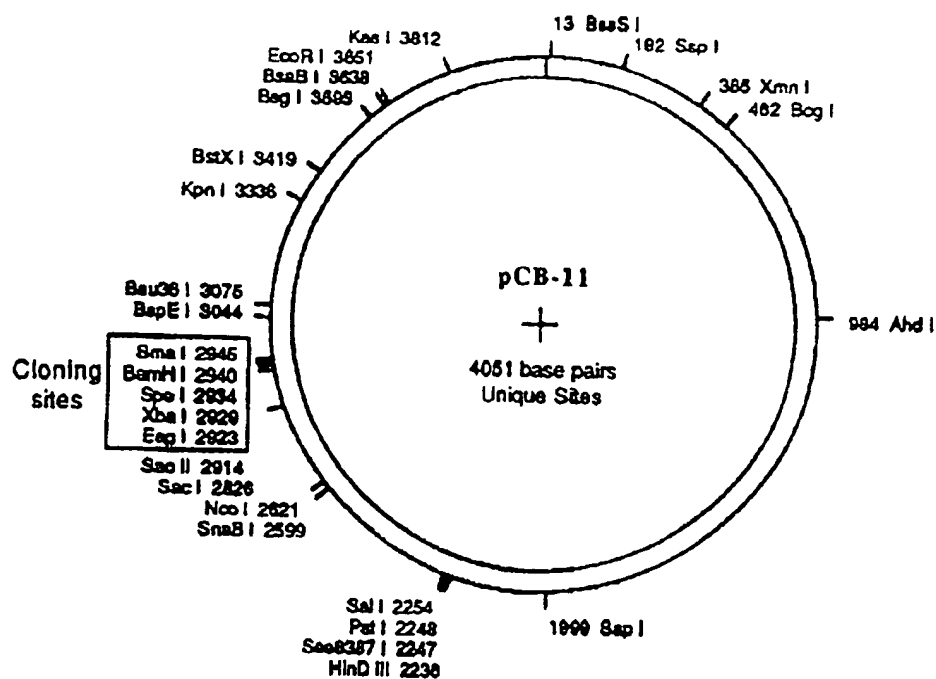
hindIII

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FIGURE 6A

[illegible]

[illegible]



2255-2920: hCMV IE1 enhancer/promoter
 2923-2951: Multiple cloning sites
 2952-3650: BGH terminator
 3651-4051 and 1-2254: pUC19

Figure 7A

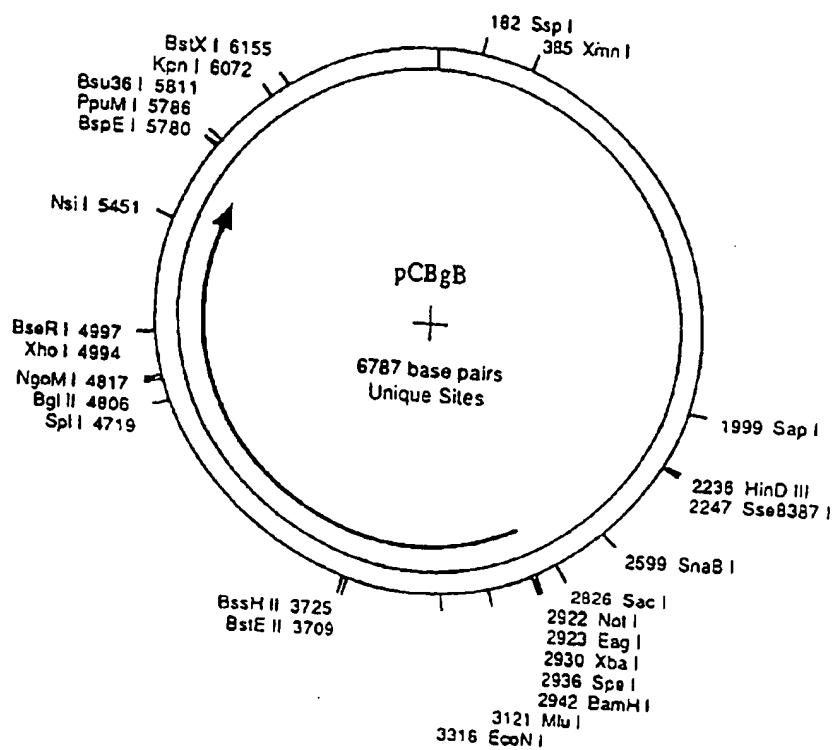


Fig. 7B

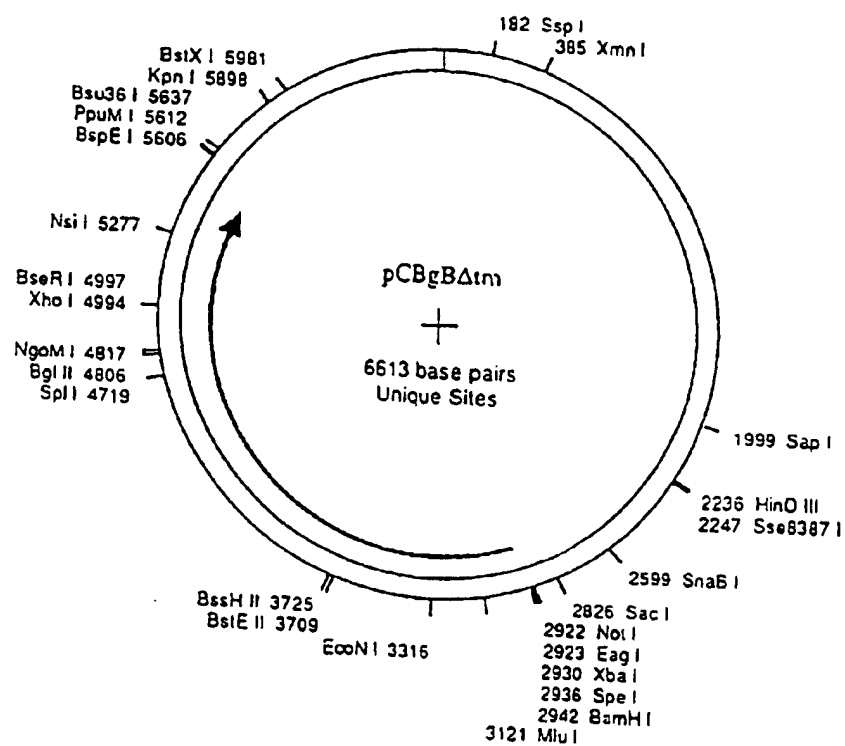


Fig. 7 C

Schematic representation of HCMV gB

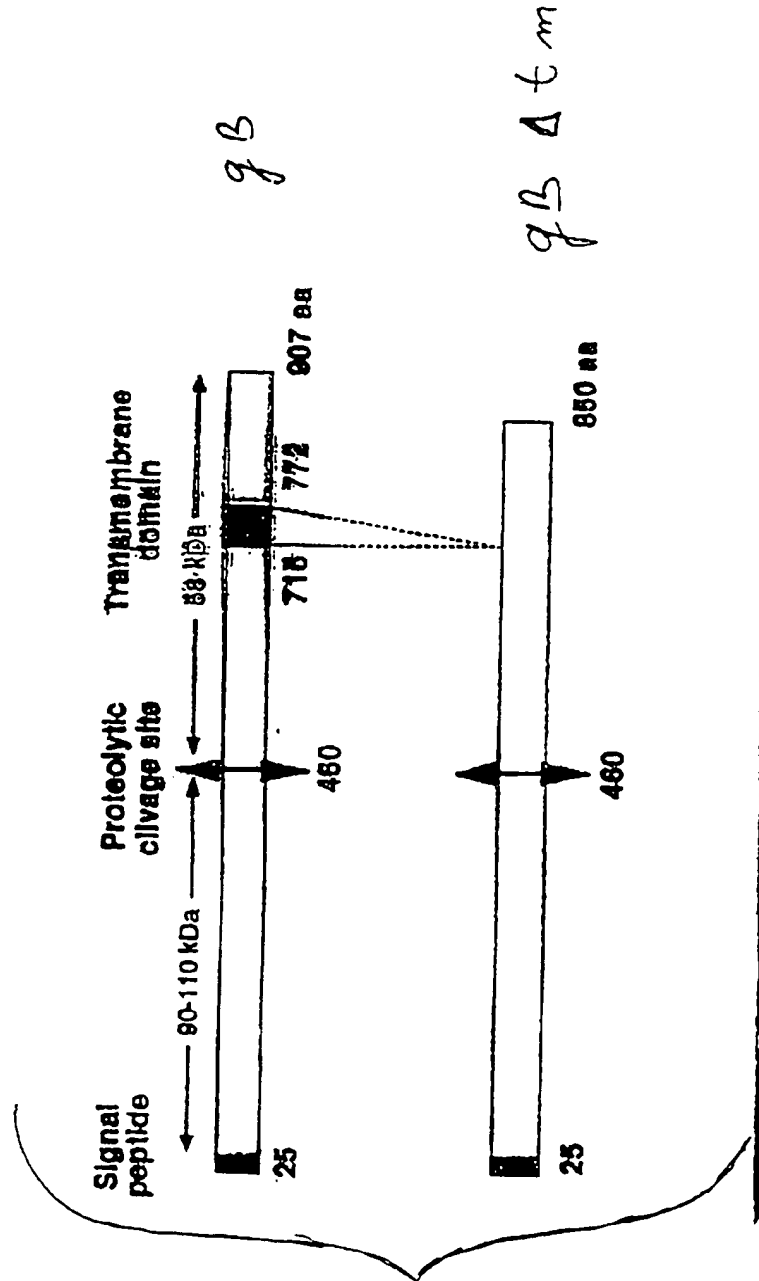


Figure 8

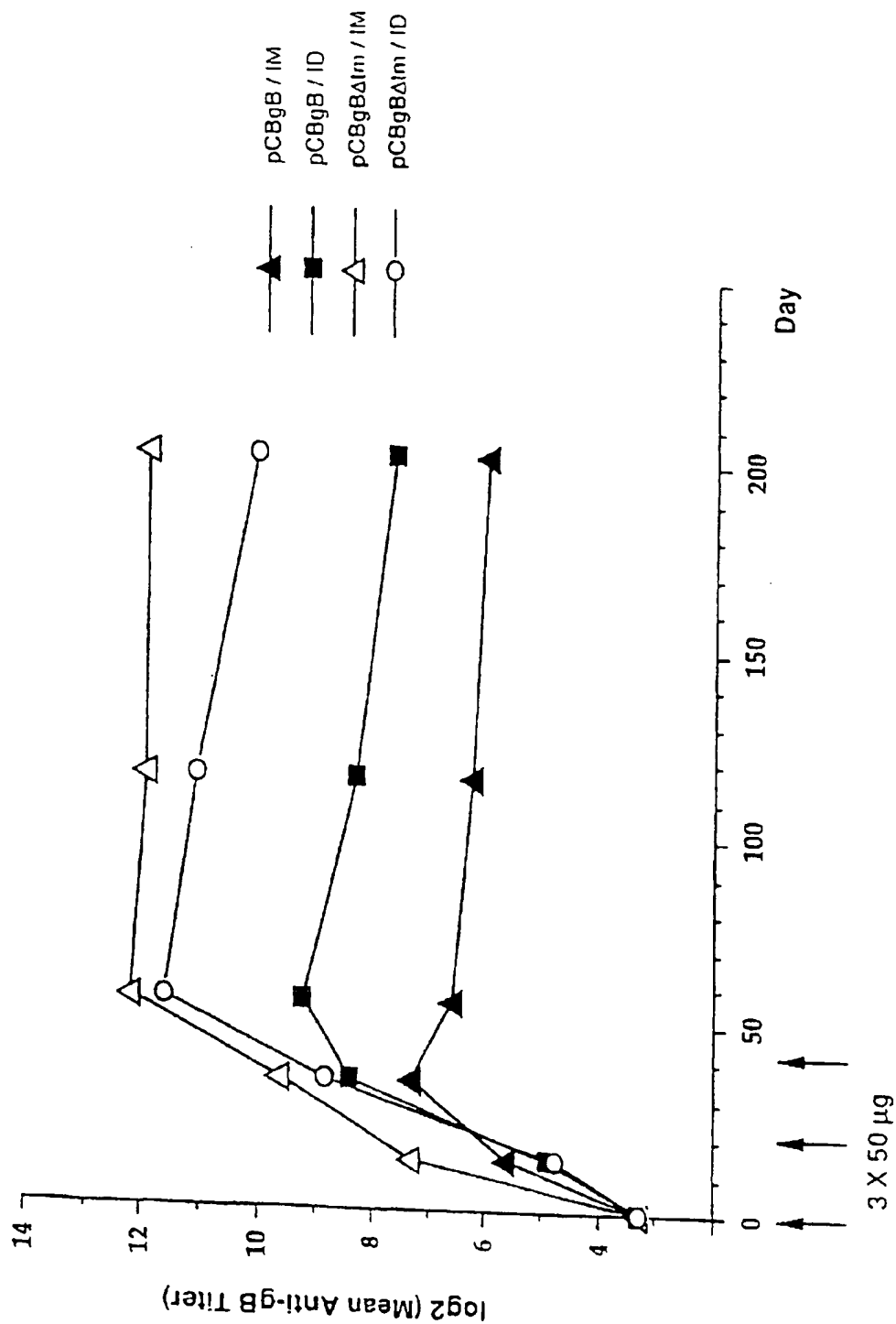


Fig. 9

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/06866

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/38 C07K14/045 A61K39/245 C12N15/86 //A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GÖNCZÖL, E. ET AL.: "Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate" VACCINE., vol. 13, 1995, GUILDFORD GB, pages 1080-1085, XP004057496	1,3,12, 16,17, 20-23, 26,27
Y	see the whole document	22,23, 26-28

	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "A" document member of the same patent family

Date of the actual completion of the international search

18 September 1997

Date of mailing of the international search report

01.10.97

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Fax (+ 31-70) 340-3016

Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

International Publication No.

PCT/US 97/06866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERENCSI, K. ET AL.: "The N-terminal 303 amino acids of the human cytomegalovirus envelope glycoprotein B (UL55) and the exon 4 region of the major immediate early protein 1 (UL123) induce a cytotoxic T-cell response" VACCINE., vol. 14, April 1996, GUILDFORD GB, pages 369-374, XP004057290 cited in the application see the whole document ---	22,23, 26-28
X	EP 0 609 580 A (CHIRON CORP) 10 August 1994 see page 8, column 1 - column 45 see examples ---	1-3,12, 16,17, 21-23, 26,27
O,X	GONCZOL, E. ET AL.: "Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate; immune response elicited in a prime/boost protocol with the glycoprotein B subunit." SCANDINAVIAN JOURNAL OF INFECTIOUS DISEASES, SUPPLEMENT 99, 1995, (110-112)., XP002041029 see the whole document ---	1,3,12, 16,17, 21-23, 26,27
A	DHAWAN, J. ET AL.: "Tetracycline-regulated gene expression following direct gene transfer into mouse skeletal muscle" SOMATIC CELL AND MOLECULAR GENETICS, vol. 21, 1995, pages 233-240, XP002041030 cited in the application ---	
A	BERENCSI, K. ET AL.: "MURINE CYTOTOXIC T CELL RESPONSE SPECIFIC FOR HUMAN CYTOMEGALOVIRUS GLYCOPROTEIN B (GB) INDUCED BY ADENOVIRUS AND VACCINIA VIRUS RECOMBINANTS EXPRESSING GB" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2507-2512, XP002026070 cited in the application --- -/--	

1

INTERNATIONAL SEARCH REPORT

International Publication No.

PCT/US 97/06866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANDE, H. ET AL.: "HUMAN CYTOMEGALOVIRUS STRAIN TOWNE PP65 GENE: NUCLEOTIDE SEQUENCE AND EXPRESSION IN ESCHERICHIA COLI" VIROLOGY, vol. 182, no. 1, May 1991, pages 220-228, XP000561310 ---	
A	GOSSEN M ET AL: "TIGHT CONTROL OF GENE EXPRESSION IN MAMMALIAN CELLS BY TETRACYCLINE-RESPONSIVE PROMOTERS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 12, 15 June 1992, pages 5547-5551, XP000564458 ---	
A	EP 0 252 531 A (BEHRINGWERKE AG) 13 January 1988 -----	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatic application No.

PCT/US 97/ 06866

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 17-28
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/06866

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